Biologically-Inspired Systems

Andreas Vilcinskas
Editor

Insect Biotechnology



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Foreword

The idea to publish this guide on latest applications, challenges and future trends in applied entomology arose in 2007 at the first International Congress on Insect Biotechnology and Industry (ICIBI) in Daegu (Korea), which was organized by the Korean Entomological Society. The rapid and multi-faceted developments in the use of insects or derivatives thereof in medical, agricultural, and industrial processes for human benefit calls for a comprehensive guide providing information about the principles behind each technology which share considerable economic potential. Insect biotechnology can be defined as the use of insects as well as insect-derived cells or molecules in medical (red biotechnology), agricultural (green biotechnology), industrial (white biotechnology), or other technological applications such as bionics which focuses on the systematic transfer of solutions from nature to the technical world. Biotechnology comprises a very broad spectrum of applications which are differentiated by established color codes, for example, "blue biotechnology" refers to technological application of marine organisms to produce food, cosmetics, medication, or new materials. By analogy, I propose the alternative term "yellow biotechnology" when insects or insect-derived cells or molecules are used in medical, pharmaceutical, agricultural, or industrial applications because the color code does not as yet include yellow and the insect hemolymph often has a yellow color.

The remarkable advances in insect biotechnology are essentially driven by scientific breakthroughs in molecular biology, particularly by the development of tools and techniques that allow genetic characterization and engineering of organisms and cells. The use of insects to produce valuables or food is not a new technology, but rather a tradition found in many human cultures. The long-term use of some insect species such as the silk moth or bees for human benefit finally resulted in their domestication similar to mammalian domestic animals such as cows, sheep, and camels. In fact, the production of silk by cocoon-spinning caterpillars of the silk moth *Bombyx mori* started in Asia more than 5,000 years ago and developed from a traditional to an industrial technology since the use of silk has been expanded beyond a raw material for manufacturing clothes to include its application in production of high-tech products such as parachutes or bullet-proof waistcoats. Bees have been exploited as a source of honey or wax for centuries. Because of the traditional use of insects in Asian countries, which are still the biggest producers of silk and

honey, the emerging competition between them in developing insect biotechnology as a highly profitable field is not surprising. For example, competition emerged between China and Japan as they independently began sequencing the genome of *B. mori*, but both campaigns were later merged resulting in successful characterization of the first lepidopteran genome.

However, based on our experiences insects may elicit negative associations because they are almost the most important competitors for human nutrition. The threat imposed by insects on agricultural production and storage of human food has plagued humanity throughout history, being manifested by locust swarms as one of the seven biblical plagues. In addition, insects were and still are the most important vectors of human disease such as plague and malaria and, therefore, account indirectly for a hundred million deaths annually. Consequently, previous research in applied entomology has predominantly focused on development of strategies and techniques to efficiently kill insects. Since it has been recognized that insectivore insects or parasitoids are important allies in fighting against pest or vector insects, and that other beneficial insects such as bees play an economically important role as pollinators, it has become apparent that sustainable and environmentally friendly approaches require the availability of selective insect control measures. The targeting of pests or vector insects has become imaginable as scientific breakthroughs in molecular biology such as RNA interference technology can be combined with expanding knowledge about insect genomes. During the writing of the chapters contributing to this book in 2009, 50 insect genome projects had either been finished or were in progress. The outcome of all of these activities in the future may be crops expressing double-stranded RNA mediating silencing expression of essential genes in pest insects without impeding non-target organisms. Additionally, other approaches such as insect transgenesis and sterile insect techniques have been proven to provide sophisticated alternative tools for targeted control of pest or vector insects. The rapidly spreading and growing knowledge about innovative approaches that meet the definition of insect biotechnology provided above clearly needs to be structured and comprehensively outlined in a textbook. Being aware that only leading experts in their respective fields are able to provide insight into the state-of-the-art in different areas of insect biotechnology and corresponding cutting-edge technologies, both the publisher and the editor are convinced that the authors selected for each chapter have properly addressed hot spots in the field.

The first part of the book focuses on the use of insects or insect-derived cells and molecules in human healthcare. Insects such as the caterpillars of the greater wax moth *Galleria mellonella* may effectively replace mammalian model hosts because they are cheaper to rear and ethically more acceptable, while others such as *Drosophila* are predestined for the study of molecular mechanisms behind human diseases like asthma. The application of insects in “red biotechnology” can be expanded beyond their use as model hosts and genetically tractable model organisms to include their value as a source of novel compounds with therapeutic potential. One impressive application of insects in medicine is highlighted in a chapter describing the development from traditional use of maggots in therapy of chronic and/or non-healing wounds to current approaches aiming at the identification of

maggot-derived molecules with therapeutic potential to generate synthetic or recombinant analogues for application in biosurgery. Insects represent the most successful group of organisms on earth regarding biodiversity, and their tremendous variety at species level may be extended to the molecular level. Taking into account that many bioactive molecules identified in insects originate from associated microbes the diversity of insect-derived compounds is virtually several orders higher. Therefore, two chapters have been incorporated which focus on molecules with therapeutic potential produced by either microbial symbionts or pathogens of insects.

The second part of the book addresses the input of insect biotechnology to modern and sustainable approaches in plant protection. "Green biotechnology" may profit from the raising spectrum of insect-derived genes encoding anti-microbial peptides whose transgenic expression has been proven to confer on crops resistance against economically important phytopathogens. Identification of species- or order-specific targets for RNA interference is the subject of a subsequent chapter describing another promising technology in plant protection which may enable selected measures against pest insects while not impeding non-target organisms.

The third part of the book focuses on industrial application of insects or insect-derived cells or molecules. Insect-derived cell lines prosper in use as heterologous expression systems for production of peptides or proteins used, for example, as vaccines in human healthcare or as enzymes mediating industrial processes in "white biotechnology." Finally, insect biotechnology encompasses the development of both novel biosensors on the basis of insect olfaction and biomimetics in various areas of technology. Far from being complete, the 13 clearly structured and simply explained expert contributions may provide an overview about current and prominent advances in insect biotechnology which will help students and researchers to broaden their knowledge and to gain an understanding of both the challenges and the opportunities behind each approach. This guide has been written in order to stimulate and accelerate the process of developing innovations in insect biotechnology. Finally, it may change the perception of insects by entomologists and nonexperts in the field in a positive manner.

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Part I
Insect Biotechnology in Medicine

Chapter 1
The Greater Wax Moth *Galleria mellonella* as an Alternative Model Host for Human Pathogens

Krishnendu Mukherjee, Eugen Domann, and Torsten Hain

Abstract Pathogenic infections are the cause of millions of deaths all over the world. Limitations in the use of mammalian models and requirements for novel strategies to counter resistant bugs have led to the emergence of insects as alternative models. Insects are widely used as feasible and convenient model systems to evaluate pathogenesis of numerous human pathogens. The fruit fly *Drosophila melanogaster* is by far the most intensively used model system to evaluate infection and developmental biology of the host in varying detail. However, the lepidopteran greater wax moth, *Galleria mellonella* is attracting increasing attention as an improved model system for several pathogens having medical significance. Among the advantages provided by insects (e.g. low rearing costs, dsRNA-mediated gene silencing, convenient injection feasibility, and ethically acceptable animal model) it is of particular importance that *Galleria* can be reared at mammalian physiological temperatures i.e. 37°C to which human pathogens are adapted and which are essential for synthesis of many virulence/pathogenicity factors. This chapter focuses on the validity and limits of using the *Galleria* model and the outcome of recent studies in which it has been used (1) as a surrogate host to study pathogenesis and virulence factors of prominent bacterial and fungal human pathogens, (2) as a whole-animal high-throughput system for testing pathogen mutant libraries, and (3) as a reliable and more "simple" organism than vertebrates to elucidate the complex molecular mechanisms of microbial pathogenesis in human-like fatal infections of the brainstem.

Keywords *Galleria mellonella* · Human pathogens · Innate immunity · Infection biology

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1.1 Introduction

Our ecosystem consists of microorganisms with varying infection potential to humans. The type of infections may have varied from time to time depending on climatic conditions, improved healthcare facilities, and food processing conditions but the impact of fatal microbial challenge in humans has always been a greater concern for medical research. Thus, research investment in the field of microbe-related infections has always been a top priority. Mammalian models like mouse, guinea pig, and rabbit have been explored to understand human infections in greater detail. Experiments have been performed to understand how a pathogen infects its host and how the host's immune machinery counteracts infections. However, in spite of detailed studies on mammalian models, the threat of microbial infections has not yet been eradicated. The vast potential of human pathogens to withstand novel anti-microbial challenges through gene mutations and expressions of virulence factors has forced an alteration in research strategies. The urge for a simpler, cost effective yet powerful model system for easy and quick detection of pathogenesis, has led to the emergence of insects to study infection biology of human pathogens.

The vast majority of the ecosystem consists of insects like fruit flies, moths, beetles etc which also share close proximity to the microbial populations present in the environment. The innate immune system of insects is thoroughly capable of maintaining microbial balance without becoming infected. It is known that their innate immune system shares similarities with the mammalian ones and thus monitoring human pathogenic infections in insects is possible as a primary screen. However, ethical restrictions associated with the use of mammalian models, their long reproductive cycles and the complex crosstalk between the adaptive and the innate immune systems limits high-throughput screening of mutant microbial strains in the mice model and have argued for the exploration of alternative model hosts such as insects (Mylonakis, 2008).

Among the insect models *Drosophila melanogaster* is the oldest and most widely used. Since its introduction as a model host *Drosophila* have been utilized in the study of complex human diseases from sepsis to Alzheimer's. The sequenced genome of *D. melanogaster* provides the advantage of conducting knockout studies especially of relevant candidate genes through RNAi-mediated gene silencing technology. The short reproductive cycles, ethical acceptance, and possibility to perform microarray-based gene analysis, makes *Drosophila* one of the forerunners as a model host for studying microbial pathogenesis.

However, apart from *D. melanogaster*, the huge potential of insect species in scientific research has not yet been properly explored. It is quite evident that the use of insects provides the possibility of new improved model systems and novel anti-microbial strategies to study human pathogenic infections in varying detail. In this hunt for new insect species as reliable model hosts for human pathogens the greater wax moth *Galleria mellonella* deserves a special mention. This lepidopteran species is found almost all over the world and is a common insect pest for honeycombs. However, in recent years *Galleria* has emerged as a reliable and robust animal model for in-vivo toxicology and pathogenicity testing. Several potential

pathogenic bacteria and fungi of medical importance have been studied with the *Galleria* model providing novel insight into their pathogenesis.

1.2 Advantages of the *Galleria* Model

The important objectives of establishing a new model system are the additional advantages that it provides unlike all other established models. Unlike *Drosophila*, the most widely accepted insect model, *Galleria* provides important advantages in studying septic infection by several human pathogens: (1) The large size of the *Galleria* larvae (2–3 cm) provides the possibility to inject desired microbial inoculums or anti-microbial therapeutics directly into the hemolymph as usually done in mammalian models (Fig. 1.1); (2) apart from septic infection studies *G. mellonella* can be used to mimic oral infections by food-born pathogens like *Bacillus cereus* (Fedhila et al., 2010); (3) large hemolymph contents (~20 μl) can easily be obtained by bleeding *Galleria* caterpillars to monitor immune-related cellular events like phagocytosis or to perform reliable proteome-related experiments; (4) and the possibility to study bacterial infections at 37°C in *Galleria* results in more reliable estimations of microbial virulence. Monitoring infection at 37°C is an important parameter to study bacterial pathogenesis since at this temperature most human pathogens express their virulence factors. (5) Recently over 70% of the whole transcriptome of *G. mellonella* has been determined by a pyrosequencing approach. Hence it is possible to develop genetic tools like RNAi knockdown of candidate genes and microarray techniques to address host-related immune responses in *Galleria* in more detail.



Fig. 1.1 Demonstrating the injection technique in *Galleria mellonella*

1.2.1 *The Greater Wax Moth* G. mellonella *as a Host for Human Pathogens*

1.2.1.1 The *Galleria* Model System for Human Pathogenic Bacteria

In recent years, the use of the *Galleria* model for studying potential human pathogens has gained increasing acceptance in the field of medical research.

Important human pathogenic bacteria like *Staphylococcus aureus* (Peleg et al., 2009), *B. cereus* (Fedhila et al., 2006), *Pseudomonas aeruginosa* (Miyata et al., 2003), *Francisella tularensis* (Aperis et al., 2007), and several human pathogenic fungi like *Cryptococcus neoformans* (Mylonakis et al., 2005), *Candida albicans* (Brennan et al., 2002), and *Aspergillus flavus* (St. Leger et al., 2000) have been successfully studied in the *Galleria* model yielding similar infection rates as observed in mammalian models like mice.

Several human pathogenic bacteria are phylogenetically related to potential entomopathogens and thus they can cause mortal infections in both insects and mammalian hosts. *Bacillus thuringiensis*, widely used in biological control of pest and vector insects worldwide, is closely related to *Bacillus anthracis*, the causative agent of anthrax and also a potential bioterrorism agent. Both bacteria have been reported to use a similar set of virulence factors, among which metalloproteinases play a predominant role (Chung et al., 2006). *B. cereus*, an emerging human pathogen and causative agent of food-born infections like gastroenteritis, is also closely related to *B. thuringiensis* and *B. anthracis* (Salamitou et al., 2000). The invertebrate model host *G. mellonella* was found susceptible to *B. cereus* infection with the involvement of virulence factors also required for mammalian infections like the transcriptional regulator *plcR*, and the flagellar protein encoding gene *flhA*. In addition, insect-specific factors such as *ilsA*-expressing internalin-like protein is required for oral infection (Fedhila et al., 2006; Bouillaut et al., 2005).

Additional recent studies have illustrated the utility of the *Galleria* model in the functional analysis of virulence factors from the human pathogenic *Enterococcus faecalis* and *Burkholderia* species. Virulent *E. faecalis*, for example, releases an extra-cellular gelitinase, GelE, that destroys defense molecules like cecropin-like anti-microbial peptide and complement component C3 both in the *Galleria* hemolymph and human serum (Park et al., 2007) whereas *Burkholderia* showed concomitant reduction in virulence and increased survival in *Galleria* following deletion of several virulence genes known to be relevant for a successful infection process in mammals (Seed and Dennis, 2008). In addition, in vivo phage therapy experiments with *Burkholderia cenocepacia* complex (BCC) infections in *G. mellonella* larvae revealed that lethal effects of a *B. cenocepacia* K56-2 infection were impaired in more than 90% of infected larvae with a single injection of BCC phage KS12. This study demonstrates the efficacy of BCC phages as anti-bacterial agents applied in the *Galleria* infection model.

Despite the obvious similarities between closely related insect and human pathogens, we should take the differences into account. For example, *G. mellonella* has been exploited to study the effects of toxins that are released via the type III secretion system (TTSS) by *P. aeruginosa*, a versatile pathogen that is capable of causing disease in plants, nematodes, insects, mice, and humans. Miyata and co-workers reported, that a deletion of the TTSS *pscD* gene of *P. aeruginosa* strain PA14 resulted in a highly attenuated virulence phenotype in *G. mellonella*, but only the gene products of ExoT and ExoU, two of four major effector proteins play a significant role in *G. mellonella* killing (Miyata et al., 2003). The high level of correlation between effects caused by the *P. aeruginosa* type III secretion system in *G. mellonella* and in mammalian tissue culture systems demonstrated the suitability

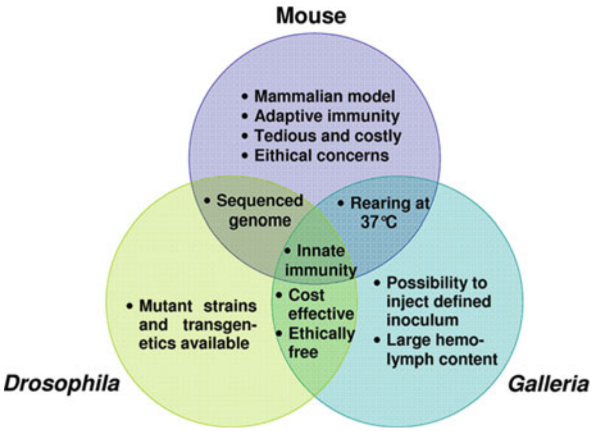


Fig. 1.2 Advantages and disadvantages of *D. melanogaster* and *G. mellonella* in comparison to *Mus musculus*

of this model (Miyata et al., 2003). In contrast, the entomopathogenic soil bacterium, *Pseudomonas entomophila*, lacks a type III secretion system (Vodovar et al., 2006) and yet were highly pathogenic to insects like *D. melanogaster*. However, the type III secretion system is obviously present in other insect pathogens such as *Photorhabdus luminescens*, which secrets toxins similar to those of *Yersinia pestis* (Joyce et al., 2006). A relative of the plaque-causing species is *Yersinia pseudotuberculosis*. A superoxide dismutase C (*sodC*) mutant of this Gram-negative pathogen showed increased susceptibility to superoxide, a key mechanism of killing in insect hemocytes and mammalian phagocytes. This mutant showed also reduced virulence in the murine yersiniosis infection model and in contrast to its wild type was unable to kill *G. mellonella* (Champion et al., 2009).

Mylonakis and co-workers used *G. mellonella* larvae as a suitable host system to study the efficacy of antibiotics against *F. tularensis*, the causative agent of tularemia, which has been considered a category A bioterrorism agent by the Center for Disease Control and Prevention in the USA (Aperis et al., 2007). In this study antibiotic therapy with ciprofloxacin, levofloxacin, or streptomycin administered before or after inoculation prolonged survival and decreased the tissue burden of *F. tularensis* in the hemocoel, whereas delayed drug treatment reduced the efficacy of antibacterials and especially streptomycin.

These studies illustrate the high potential of *G. mellonella* as a high-throughput whole-animal system to evaluate antimicrobials before they are tested in mammalian models (Fig. 1.2).

1.2.1.2 Use of the *Galleria* Model System to Study Septic Infection by *Listeria* – A Case Study

Pathogenomics of *Listeria*

Listeria are a group of Gram-positive facultative anaerobes, comprising eight species, namely *Listeria monocytogenes*, *L. innocua*, *L. seeligeri*, *L. ivanovii, L. welshimeri*, *L. grayi, L. marthii*, and *L. rocourtiae* (Hain et al., 2007; Graves et al.,

2010; Leclercq et al., 2009). The pathogenic strain *L. monocytogenes* is responsible for infections in humans and *L. ivanovii* is pathogenic to ruminants like sheep. Other *Listeria* species were not reported to be involved in fatal listerial infection. *Listeria* anaerobes are ubiquitously distributed in the environment and are resistant to extreme food-manufacturing processes like high temperature, pH and salt concentration. The human pathogen *L. monocytogenes* causes infections called listeriosis in mammals which later develop into fatal infections like meningitis, meningoencephalitis, and septicemia (Midelet-Bourdin et al., 2006). Recently *L. monocytogenes* contamination in a food plant in Canada resulted in 23 casualties along with 57 confirmed cases.

It is known that the virulence of *L. monocytogenes* has been linked to a 9.6-kb pathogenicity island designated *vgc* (*vi*rulence *g*ene *c*luster) that comprise six genes encoding its major virulence determinants. These include (1) *prfA*, a master regulator of many known listerial virulence genes, (2) *hly*, encoding listeriolysin, a hemolysin required for bacterial escape from the host primary vacuole to the host cytoplasm, (3) two phospholipases denoted *plcA* and *plcB*, which facilitate lysis of host cell membranes, (4) *actA*, a surface bound protein that directs polymerization of host cell actin and is required for intra-cellular motility, and (5) *mpl*, a metalloproteinase which is thought to work together with *plcB* to facilitate cell-to-cell spread (Hamon et al., 2006). Mammalian models like mice have successfully been used to study septic *L. monocytogenes* infection in great detail. The role of major virulence factors like the 9.6-kb virulence gene cluster or the virulence associated genes like *mprF* or *dlt* contributing to *L. monocytogenes* virulence has been elucidated in these models (Thedieck et al., 2006). However, identification of novel virulence factors requires large-scale screening of *Listeria* mutants which is costly and ethically debatable in the mouse model. Moreover, the pathogenic potential of *Listeria* is further enhanced by a growing number of strains resistant to anti-microbial compounds particularly to antibiotics (Linnan et al., 1988, Davis and Jackson, 2009).

1.2.1.3 Correlation of Mammalian Infection in *Galleria*

Recently, estimation of *Listeria* virulence was reported in the invertebrate model host *G. mellonella* correlating infection in mammals (Mukherjee et al., 2010). Septic infection with the human pathogenic *L. monocytogenes* resulted in higher mortality of the *Galleria* caterpillars whereas other *Listeria* species like *L. innocua, L. seeligeri*, *L. welshimeri*, and *L. grayi* failed to cause mortal infections, similar to the mammalian models. *L. ivanovii*, responsible for pathogenic infections in ruminants, was also pathogenic to *Galleria*. The different serotypes of *L. monocytogenes* like 1/2a, 4a, 4b, 4c, and 4d also provided similar pathogenic evidence in the *Galleria* model to that known from experiments in mice. The 4b serotype is commonly associated with severe *Listeria* outbreaks and also possesses high cellular invasive power following increased mortality rates in mice after infection. In *Galleria* the 4b serotype was highly pathogenic causing the highest morality of the caterpillars in comparison to the pathogenic 1/2a. Serotypes like 4a, 4c, and 4d that are

less virulent in mammalian infection models also showed virulence attenuation in *Galleria* (Mukherjee et al., 2010).

Apart from re-characterizing the pathogenic potentials of different *Listeria* species and *L. monocytogenes* serotypes, *Galleria* was successfully employed to study pathogenicity-related genetic diversities contributing to *L. monocytogenes* virulence. The virulence gene cluster (vgc) comprising six virulence associated genes, namely *prfA*, *hly*, *plcA*, *actA*, *mpl*, and *plcB* was found to be the main regulator of *L. monocytogenes* virulence in *Galleria*. In contrast, chitinases from *L. monocytogenes* were recently found to be capable of hydrolyzing α-chitin from arthropods, which may be important for infection of invertebrates (Leisner et al., 2008). Moreover, *L. ivanovii*, which is specifically pathogenic to ruminants, was clearly less pathogenic in the *Galleria* model than *L. monocytogenes* but nevertheless still demonstrated a significant difference in mortality compared to the non-pathogenic *L. innocua*. Genes related to bacterial survival in the host like the hexose phosphate transporter *uhpT*, which is required for efficient growth and survival of *L. monocytogenes* in infected vertebrate cells were also found to be essential for *Galleria* infection (Mukherjee et al., 2010). This indicates that apart from being a robust model system to differentiate different *Listeria* species, serotypes, and mutants based on their virulence potentials, the energy-rich phosphorylated sugar derivatives in *Galleria* are also an essential source for *L. monocytogenes* survival in invertebrates. From the results mentioned above, it can also be concluded that insects like *Galleria* may represent natural reservoirs of the environmental pathogen *Listeria*, which is an example for the survival of other human pathogens in other invertebrates of the ecosystem.

1.2.1.4 Cellular Responses in *Galleria* Following *L. monocytogenes* Infection

Following the entry of *L. monocytogenes* in mammals, immune cells like macrophages and dendritic cells form suppurative granulomas to control listerial infection of tissues and organs. Granuloma-like structures were also observed in *Galleria*, indicating similar involvement of hemocytes namely the granulocytes and plasmatocytes in controlling *L. monocytogenes* infection of tissues (unpublished data). The infection mechanism of *L. monocytogenes* includes polymerization of host cell actin, which is required for intra- and inter-cellular locomotion. By the expression of the virulence gene *actA*, *L. monocytogenes* induces actin tail formation in mammalian macrophages. A similar mechanism of actin tail formation was also observed in the hemocytes of *Galleria* infested with *L. monocytogenes* strain EGD-e (Fig. 1.3).

1.2.1.5 Systemic Induction of Anti-Microbial-Related Immune Genes in *L. monocytogenes*-Infested *Galleria*

Complementing the cellular defense mechanisms, *Galleria* is capable of synthesizing a broad spectrum of anti-microbial peptides in response to septic injury (Seitz et al., 2003; Brown et al., 2009). *L. monocytogenes* infections induce considerable

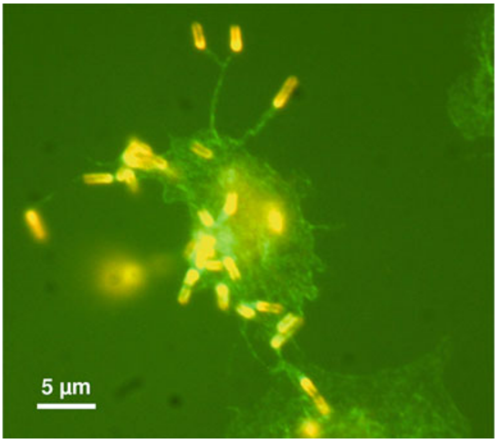


Fig. 1.3 Immunofluorescent staining of *Galleria* hemocytes infected with the human pathogenic *L. monocytogenes*. *L. monocytogenes* cells were stained using *actA* antibodies (resulting in *red fluorescence*) and the host actin of hemocytes was stained using Alexa-phalloidin (resulting in *green fluorescence*). Note *Listeria* spreading throughout the cytosol of the hemocyte and actin tails at the poles of some of the bacteria are visible

expressions of anti-microbial-related immune genes like lysozyme, galliomycin, gallerimycin, and IMPI in *Galleria* (Mukherjee et al., 2010). Administration of immune elicitors like heat-killed *Listeria* prior to lethal challenge of *L. monocytogenes* resulted in early induction of lysozyme, galliomycin, gallerimycin, and IMPI in *Galleria*, thus providing resistance against *Listeria*. However, apart from these selected studied expressions of anti-microbial peptides, the hemolymph of immune-induced Galleria is expected to produce a host of other unknown anti-microbial peptides. Interestingly, the hemolymph samples from immune-activated *Galleria* inhibited the growth of *Listeria* over culture plates (unpublished data). This indicates that *Galleria* possesses a rich source of anti-microbial peptides with high therapeutic potential against human pathogens like *L. monocytogens*.

1.3 *Galleria* as a Model System for Human Pathogenic Fungi

Invasive fungal infections are the cause of mortality among immunocompromised individuals. Economic investments in relation to invasive fungal disease corresponds to $2.6 billion annually in the United States alone (Wilson et al., 2002). Treatment of such infections is extremely difficult with the limited availability of anti-fungal drugs without host toxicity and with the emergence of multi-drug-resistant fungal strains. Apart from mammalian models the use of invertebrates like *G. mellonella* provides valuable insights in regard to countering fungal infections. Besides medically relevant human pathogenic bacteria, *Galleria* has been developed as a reliable model for human pathogenic fungi like *Aspergillus*, *Candida*, *Cryptococcus* etc.

Among the potential fungal pathogens, *Aspergillus* is of great medical interest. *Aspergillus fumigatus* is the primary causal agent of invasive aspergillosis and reigns as the most deadly mold, with mortality rates of up to 90% (Singh and Paterson, 2005). Utilizing *Galleria* models, the role of gliotoxin in the virulence of human pathogenic *A. fumigatus* has been elucidated (Reeves et al., 2004). The virulence of *A. fumigatus* against *Galleria* caterpillars depends on the stage of conidial germination. Non-germinated conidia are phagocytosed by larval hemocytes and thus fail to confer infectious attributes to the host, whereas germinated conidia are not phagocytosed and confer cytolytic attributes to the hemocytes (Renwick et al., 2006). Melanization in *A. fumigatus* is a known virulence factor in mammalian models. Mutations in the six-gene cluster required for DHN-melanin biosynthesis caused enhanced mortality of *Galleria* compared to the wild-type strain. The mammoth induction in immune response by increased exposure of pathogen-associated molecular patterns or PAMPs may cause self-damage in *Galleria* resulting in mortality following infection with *A. fumigatus* mutants having defects in melanin biosynthesis (Jackson et al., 2009). This also highlights the limitations of using insect models for elucidating the pathogenicity of *A. fumigatus* strains in mammals, but also indicates the importance of understanding the innate immunity of the insect host following fungal challenge. Another human pathogenic fungi, *C. neoformans*, associated with morbidity and mortality in immune-deficient patients like HIV-positive patients and transplant recipients, showed similar virulence in *Galleria* models. Several virulence genes of *C. neoformans* like *CAP59*, *GRA1*, *RAS1*, and *PKA1*, which are required for mammalian virulence, were found to be important for mortality of *Galleria* (Mylonakis et al., 2005). The importance of septins in *C. neoformans* development and virulence at 37°C was reported in the *Galleria* model (Kozubowski and Heitman, 2009).

C. albicans is the fourth most common cause of hospital-acquired infectious disease and the primary cause of systemic candidiasis, with mortality rates approaching 50% (Pfaller and Diekema, 2007). The virulence attributes of human pathogenic *C. albicans* in mice mirrors the situation found in the *Galleria* model. Even pre-exposure to this human pathogenic yeast protects *Galleria* caterpillars from subsequent lethal infection with *C. albicans* by increased expression of anti-microbial peptides (Brennan et al., 2002). The role of Heat shock protein 90 (Hsp90) in anti-fungal resistance has been quite explicitly demonstrated in the *Galleria* model. The therapeutic potential of anti-fungal compounds like azole, fluconazole (FL), with clinically relevant Hsp90 inhibitors that are structurally related to the natural product geldanamycin (GdA) in fungal diseases was explored using *Galleria*. At drug exposures with minimal activity on their own, the Hsp90 inhibitors 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) dramatically increased the efficacy of FL against *C. albicans*. In the *G. mellonella* model, combination therapy with GdA and CS improved survival of larvae with *A. fumigatus* infections that were lethal, despite monotherapy with either agent.

1.4 Conclusion

It is quite evident that future scientific research will rely increasingly on the alternative infection models to combat human pathogens. Especially this era of bio-medicine dominated by the growing number of multi-drug-resistant strains and a lack of new antibiotics for treatment, advocates for improved anti-infective therapies. In this regard the use of *G. mellonella* will provide novel insight into the infection biology of human pathogenic bacteria, fungi, viruses, and protozoa. Moreover, the rich anti-microbial peptides present in the *Galleria* hemolymph are a promising source for novel therapeutics against various human pathogens.

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Chapter 2
Fruit Flies as Models in Biomedical Research – A *Drosophila* Asthma Model

Thomas Roeder, Kerstin Isermann, Christina Wagner, and Christine Warmbold

Abstract Inflammatory diseases of the lung such as asthma and COPD show an increasing prevalence in western countries. Although these diseases are typically complex ones, they have an important genetic component. Genome-wide association studies have provided us with a comprehensive list of asthma susceptibility genes that will be extended substantially in the near future. To identify the role of these genes in the physiology and pathophysiology of the lung genetically tractable model organisms are indispensable. The inherent limitations of present models such as the mouse represent a constant urge for novel, complementary models. The fruit fly *Drosophila* has the potential to close this gap, as it might prove to be extremely helpful in the translation process from genetics to biological function. Except for those asthma susceptibility genes associated with adaptive immunity, we found unequivocal homologues for all of them in the fly genome. In addition, most of these candidates are indeed expressed in the airway epithelium and/or in other organs relevant for asthma, namely the blood cells and the brain. A majority of them are regulated upon airway infection in the *Drosophila* airway epithelium pointing to an important role in airway immunity and development of asthma-like phenotypes in the fly. These surprising similarities at the molecular level, in combination with the unmatched technical possibilities available to researchers using *Drosophila* should complement murine models in various aspects of asthma research. Biomedical research critically depends on animal models to understand the molecular basis underlying the pathogenesis of human diseases and to provide systems for developing and testing new therapies. Despite the supremacy of murine models, other model organisms are able to provide new and relevant information. All these organisms, including mice, are characterized by a set of features allowing us to categorize them as real model organisms. In addition to the sequenced genomes, the short life cycles, and the similarities with human genes/proteins, the ease of genetic manipulation is of prime importance. Among the limited number of well established and generally accepted model organisms (yeast, *C. elegans*, *Drosophila*, zebra fish, and

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mice), the fruit fly *Drosophila* is the only insect. It is the oldest model organism and was introduced almost a century ago by Thomas Hunt Morgan. Sequencing and analysis of its genome revealed a completely unforeseen degree of similarities with our own genome. More than 60% of all human disease genes have homologous counterparts in the fly (Fortini et al., 2000), which led to the development of a special database listing all these candidate genes (Chien et al., 2002). Among the first studies that utilized *Drosophila* with the goal to learn more about the molecular events underlying these diseases are those that established corresponding models for neurodegenerative diseases (Feany and Bender, 2000). In addition to this Parkinson model, very informative models of Huntington's and Alzheimer's disease have been established (Chan and Bonini, 2000), which triggered a great number of follow-up studies. In the last years, various different *Drosophila* disease models have been introduced (Bier, 2005). Only two out of a plethora of corresponding studies should be mentioned, i.e. models for the analysis of cardiac diseases (Wolf et al., 2006) and diabetes (Baker and Thummel, 2007). For the unprejudiced reader it may be hard to understand why *Drosophila* should be that well suited. The fruit fly is central to all model organisms; its organization is much simpler than in mice. *Drosophila* is simple enough to function as an easy to use model, but major organs, physiological processes, and behaviors are very similar to those found in men. This is of special importance, because it makes comparisons between men and flies much easier than comparisons between men and worms or even between men and yeast. The major question is what makes *Drosophila* so special? It is a combination of a vast amount of knowledge that has been accumulated during the last century and the availability of countless technical opportunities to manipulate the fly that are beyond comparison.

Keywords *Drosophila* · Asthma · Airway epithelium · NF-kB · Chronic inflammation

2.1 What Is Asthma?

Asthma is the most common chronic inflammatory disease of the lung. Like most inflammatory diseases, asthma is a complex disease, meaning that different intrinsic and extrinsic factors contribute to pathogenesis. Asthma has a definite genetic component and family and twin studies revealed that the risk of developing asthma is increased several fold in first-degree relatives of asthma patients (Holloway et al., 2008). Positional cloning as well as candidate gene studies led to the identification of numerous asthma susceptibility genes. As some of them don't fit into the generally accepted view of asthma pathogenesis, it has been suggested that new and unexpected pathways are involved in asthma development, increasing the number of potential targets for pharmacological intervention (Holgate and Polosa, 2008).

The large number of completely new generations of genome-wide association studies performed with large and well-defined cohorts of patients that are currently

being carried out will provide us with an almost complete set of asthma susceptibility genes. The usefulness of this rich source of information is currently limited by our inability to understand the connection between genes and disease development, at least for the majority of them. In asthma, the situation is obviously similar to all other complex diseases: the identification of susceptibility genes is the beginning and not the end of the effort. The only way to close this gap is to use model organisms that are thus of paramount importance to elucidate the relevance of these genes and genetic variations. Transgenic mice, including "humanized" mice, are currently the sole genetically tractable models used successfully in asthma research (Finkelman and Wills-Karp, 2008). Although they are very useful, transgenic and mutant mice have drawbacks that obscure a direct analysis of the expected phenotypes. This holds true especially if genetic redundancy and developmental plasticity are important issues. In addition, it is technically not trivial to produce mice tailored to the needs of lung physiologists, because the lung is a very complex organ with numerous resident but also motile cell types. To supplement this technical resource, other animal models, especially those which have a far lesser complexity, are mandatory.

2.2 *Drosophila* in Asthma Research

The fruit fly has gained acceptance as a model organism to study aspects of human diseases, but asthma is surely one disease that doesn't initially spring to mind. Flies are lacking in most characteristics thought to be highly relevant for asthma, such as all aspects of adaptive immunity including IgEs and T-cells. In addition, outcomes of the disease such as wheezing and airway hyper-responsiveness have not been observed in flies. This view changed dramatically in the last few years, simply because it became apparent that other factors are indispensable for disease development. Among these findings is especially the insight that epithelial cells play a central role in asthma pathogenesis (Hammad et al., 2009; Holgate, 2007). Apparently, they orchestrate most adverse effects associated with this disease in a yet not well-understood way. Another central aspect relevant for asthma development is the innate immune system and especially the innate immune system within the airway epithelial cells. It became clear that those innate immune signaling pathways that converge on activation of NF-kB factors are of central importance for various aspects of disease development and progression (Broide et al., 2005; Pantano et al., 2008). Both newly identified hallmarks of asthma pathogenesis brought the fly back into focus, simply because their contribution to disease development can be studied easily in the fly (Lemaitre and Hoffmann, 2007; Roeder et al., 2009).

Parallel to these developments, another set of asthma-related research changed the way the disease was looked at significantly. Genetic research has revealed a number of asthma susceptibility genes (Vercelli, 2008). In addition, with the development of genome-wide association studies, the roles of many more candidate genes

in asthma will be uncovered. Surprisingly, most of these asthma susceptibility genes have nothing to do with adaptive immunity. Instead they are associated with innate immunity and with ways to ensure epithelial integrity. Susceptibility genes that can be classified into either of these categories (innate immunity and epithelial integrity) comprise approximately 60% of the total. Except for those associated with adaptive immunity, we and others found unequivocal orthologues for all of them (Roeder et al., 2009). Most asthma-related genes are expressed in the airway epithelium of the fly. Even more interestingly, some are regulated upon airway infection of the *Drosophila* airway epithelium, pointing to an important role in airway immunity and development of asthma-like phenotypes in the fruit fly (Table 2.1).

As already mentioned, flies exclusively depend on their innate immune system, because they do not possess an adaptive immune system. This seeming disadvantage may develop into a significant advantage, because it allows for an exclusive view of the role of innate immunity under different physiological conditions. In mice, it is a very complex task to distinguish between responses of the adaptive and the innate immune system, because they are highly interconnected. In the absence of adaptive immune components, an unveiled view of the role of innate immune systems is possible. The airways of the fly (tracheae) have a simple architecture,

Table 2.1 Asthma susceptibility genes and their homologues in *Drosophila*

Asthma gene	*Drosophila* homologue	Genomic resources
ACE	ANCE-family	RNAi
ADAM33	Neu3 (CG7649), mmd (CG42252)	all: KO, RNAi
ADRB2	Oct2ß-like	KO, RNAi
CCL11, CCL5	upd1-3	KO, RNAi
CD14	CG5195	RNAi
CMA1	trypsin29F, snk	RNAi; KO, RNAi
DPP10	ome, CG17684	both: KO, RNAi
FLG	CG1433 Atu	KO, RNAi
GPRA	CcapR, AlCR2, TakR86C	all: KO, RNAi
GSTM1	GST-D family	all: KO, RNAi
GSTP1	GST-S1	KO, RNAi
GSTT1	GST-E family	all: KO, RNAi
MMP9	DmMMP1	KO, RNAi, OE
NAT2	aaNAT-1	KO, RNAi
NOS	dNOS	KO, RNAi, OE
ORMDL3	dORMDL	RNAi
SPINK5	serpins	KO, RNAi
STAT6	STAT92	KO, RNAi, OE
TBXA2R	CG7497, OAMB	KO, RNAi; KO, RNAi, OE
TGFb1	activin, maverick, gbp	RNAi, - ; RNAi
TLR2, 4, 6, 10	Toll receptors	all: KO, RNAi,
TNF	eiger	RNAi

Listed are the orthologues of canonical asthma susceptibility genes. The genomic resources available have been extracted from the VDRC (Dietzl et al., 2007) or flybase (www.flybase.org)
-: No resources available.

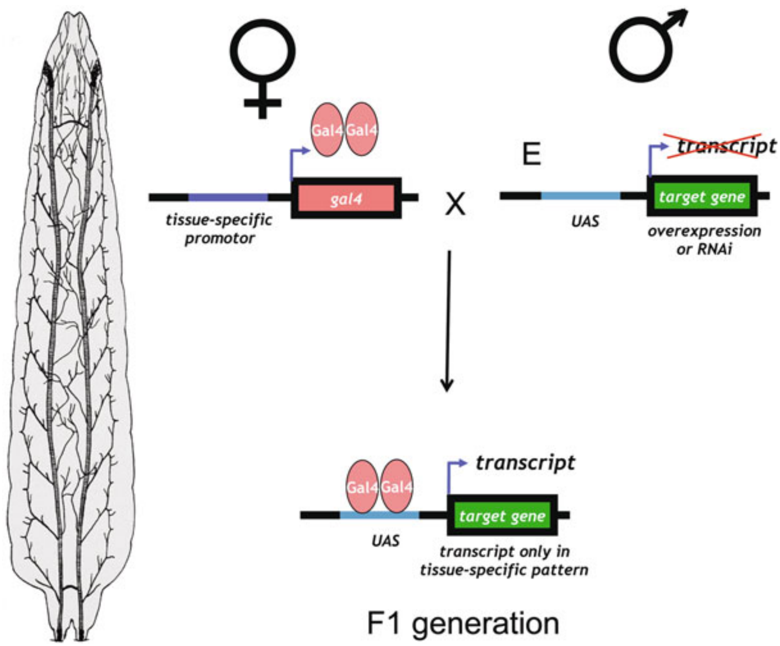


Fig. 2.1 A schematic description of the tracheal system of a third instar larvae (modified after Ruehle, 1932) shows the overall organization of the tracheal system. On the *right side*, the Gal4/UAS is depicted schematically. Gal4 flies are crossed with UAS flies (driver with effector) to yield the desired condition in the F1 generation, meaning that the gene of interest is overexpressed or silenced in the region of interest

made of interconnected tubes terminating in a multitude of fine blind-ending structures (Fig. 2.1). In its organization with primary, secondary, tertiary, and terminal branches, it resembles the overall design of the human lung (Affolter et al., 2003; Ghabrial et al., 2003; Whitten, 1957). Trachea are composed of epithelial cells only, forming a single cell layer around the central airspace. Although of low complexity, it is a highly effective structure sharing numerous physiological similarities with our own lung.

Immunological studies with epithelia of the fly revealed that these non-professional immune competent tissues are able to launch an immune response if confronted with pathogens or pathogen-associated molecular patterns (Ferrandon et al., 1998; Tzou et al., 2000). All epithelial cells have this capacity (Wagner et al., 2008). A detailed analysis of the signaling pathways present in the fly's airway epithelium and the pattern recognition molecules revealed that only one pathway converging on NF-kB activation, the IMD pathway is operational in these tissues. In addition, the majority of pattern recognition molecules that has the fly to its hands are present and functional there. It should be mentioned that other signaling pathways with relevance for immune reactions, including the JAK/STAT- and JNK-pathway, are functional in these cells (Wagner et al., 2008).

2.3 Infection and Ectopic Activation of the Immune System Induce Asthma-Like Phenotypes

The epithelial immune system of the fly's airway epithelium can react to an infection with bacteria or fungi with the expression of anti-microbial peptide genes. This response characteristic is only a small part of the entire response repertoire of this epithelium. Upon prolonged and very strong activation of this epithelial immune response, another phenotype becomes apparent that shows striking similarities to the situations observed in patients with a severe asthma phenotype. Those parts of the tracheal system that react most strongly to an airway infection show significant airway remodeling. The remodeling of airways is one major hallmark of chronic asthma. In the fly, the most conspicuous sign of remodeling is a several fold increase in epithelial thickness. This is accompanied by the production of new airway epithelial cells, something that usually does not occur during normal life in the fly. Taken together, these cells become meta- and hyperplastic, thus showing hallmarks of airway remodeling upon a prolonged strong activation of this very special type of immune system (Wagner et al., 2009). One major reason for this unprecedented type of reaction may be that these cells forcefully try to obviate apoptosis. Following infection highly potent apoptosis-inducing genes are activated, including the autophagy-specific kinase 1 gene (Atg1), whose overexpression itself is sufficient to induce apoptosis. Activation and overexpression of dFoxo appears to be a way to enable survival under these hostile conditions, which in turn, may be the major reason for the observed phenotypical variations.

2.4 What Has the Fly to Offer?

The fly offers some very intriguing experimental advantages that are not shared by other model organisms. Numerous deletions and far more than 12,000 transposable element insertions are readily available for creating genomic mutations. Transgenesis is very easy and a number of corresponding vectors are also available and some of them have been developed that capitalize on *P* element, phiC31 and recombineering technologies (Bellen et al., 2004; Venken and Bellen, 2007; Venken et al., 2009). Among the most useful tools is the binary GAL4/UAS-system invented more than 15 years ago (Brand and Perrimon, 1993). It relies on the yeast transcription factor GAL4 and the corresponding DNA binding region UAS that constitutes an extremely versatile construction kit. Gal4/UAS systems conveniently allow overexpression or silencing of genes of interest wherever the researcher requires it. Both components (Gal4 and UAS) are segregated into different fly lines, the so-called driver and effector lines. Driver lines (Gal4-lines) determine the temporal and spatial restriction of expression. Effector lines (UAS-lines) determine what is expressed. A plethora of different driver (Gal4) lines are available, enabling us to address almost every tissue in the fly separately. On the other hand, effector lines are also available (RNAi-lines, see below) or can be generated easily by producing corresponding flies carrying a construct where for example overexpression of a certain

gene can be achieved because it is under transcriptional control of UAS elements. Crossing these two types of lines activates this system in the entire F1 generation (Fig. 2.1). This simple construction kit architecture allows production of hundreds of different experiments with a very limited number of fly stocks, simply by means of combination. As the parental strains are usually not impaired, researchers can induce silencing of various genes of interest in one organ or overexpression of another gene in a different set of organs simply by crossing the corresponding lines.

Regarding asthma, the airway epithelium is obviously the most important target. For this tissue, some drivers are available that direct expression into the entire set of airway epithelial cells or into only well-defined subsets of them. Thus, these driver lines allow a well-orchestrated overexpression or gene-silencing in the entire airway or in only functionally well-defined parts of it.

A recent refinement of this extremely versatile method allows additional temporal control of expression. The so-called TARGET system (McGuire et al., 2003) includes a temperature-sensitive repressor of GAL4, named GAL80ts. It represses expression under restrictive conditions (19°C). Shifting to the permissive temperature (29°C) inactivates the temperature-sensitive repressor, thus releasing GAL4 from its inhibition, which leads to expression of the target gene. Usually Gal80ts is expressed under control of the ubiquitously active tubulin promotor, thus enabling effective inhibition in all organs at almost all time points. The process of Gal80ts inhibition is reversible and thus, returning to the restrictive temperature can terminate Gal4-mediated expression. Such a TARGET expression system has been established for the most specific airway epithelial driver, ppk4-GAL4. This versatile and simple system enables the researcher to exclude developmental effects of overexpression or silencing and to separate transient from chronic effects simply by choosing an appropriate activation regime.

To complement the huge number of genomic knock-outs and corresponding insertion lines that allow an easy production of gene-specific knock-outs, Barry Dickson's group completed the great endeavor of producing RNAi lines for all annotated *Drosophila* genes (Dietzl et al., 2007). Fortunately, they provided this collection to the *Drosophila* community. Combination of these publicly available lines with the GAL/UAS system allows efficient gene silencing in most parts of the organism or in an organ-specific fashion, depending on the GAL4 driver that is utilized. This unique resource enables even large-scale studies at reasonable prices and in reasonable time frames.

Another experimental approach is the production of "humanized" flies carrying transgenic arrays of the corresponding human genes, or the genetic variations of interest. The production of these fly strains has great potential and is much simpler and less time consuming than making sophisticated transgenic mice. As mentioned previously, comprehensive studies are possible with a very small number of stocks, due to the binary nature of the GAL/UAS system that allows free combination of driver and effector lines (Brand and Perrimon, 1993). The GAL4/UAS system is only one of the many tools available to *Drosophila* researchers. Many other extremely powerful techniques exist that maximize the abilities of what can

be studied in the fly. It is impossible to summarize all of them, but the reader is referred to additional reviews for more detailed discussion of recent developments in the field of *Drosophila* genetics (Bellen et al., 2004; Venken and Bellen, 2007; Venken et al., 2009).

2.5 What Is the Greatest Potential of *Drosophila* in Asthma Research?

As already mentioned, more asthma susceptibility genes are involved in innate immune responses or in maintaining epithelial cell integrity than in processes associated with adaptive immunity. Innate immune signaling within these epithelial cells is necessary and sufficient for experimental disease induction, either in mice or flies. As already mentioned, the central position of epithelial cells for orchestration of for example airway remodeling has been emphasized in recent physiological studies. The identification of these two major determinants of asthma development, the airway epithelial cell and the innate immune system, makes the fly very attractive as it is well suited to study the roles of both of them.

To show that *Drosophila*, with all its advantages, can develop into a model for asthma research, we focused on the canonical set of asthma susceptibility genes and tried to find homologues in the fly (Roeder et al., 2009). A smaller group of these genes, associated with adaptive immunity, have no counterparts in the fly. These genes include: IL-4, IL-10, IL-13, IL-18, IL-4R, CC16, CTLA4, HLA-DBP1, HLADQB1, HLA-DRB1, LTA, LTC, and FCERIB. For all other candidates, we found orthologues in the fly genome. This analysis is summarized in Table 2.1. Most of these orthologues are indeed expressed in the airway system of the fly and some of them (the majority) are regulated following infection or ectopic manipulation of the IMD-signaling pathway. For most of these *Drosophila* orthologues, informative genetic material is available comprising conventional knock-out strains, those that allow tissue-specific gene silencing, and additionally in some cases those that allow tissue-specific overexpression (Table 2.1).

Obviously, the methods to manipulate the airway system of the fly or orthologues of asthma susceptibility genes within this tissue are copious, but what about simple read-outs? Identification of disturbances within the tracheal system can be monitored using two very simple but nevertheless informative approaches. The transparency of the larvae allows a visual evaluation of the tracheal structure such that functional impairments that are often caused by liquid-filled parts of the tracheal lumen are easily identified. A physiological test of the operational capability of the tracheal system takes advantage of the normal behavior of the larvae. Healthy animals are usually buried in their food, but upon oxygen deprivation, they move to the surface. This is also observed in larvae that show impairments of the tracheal system. This behavior can be quantified (time to leaving food or percentage of larvae showing this behavior), thus giving a measure that provides information about the supply of oxygen to tissues.

The reason for choosing a seemingly simple model organism is that one hopes to be confronted with a significantly reduced complexity of the genomic situation, meaning with much lesser redundancy (number of genes of a certain gene family) and with much lesser plasticity (meaning that genes can take the role of a deleted one). Both aspects are obviously on the side of the fly. Usually, gene families that have numerous members in mammals are comprised of fewer or sometimes only one member in the fly, which is exemplified by the matrix metalloproteinases (MMPs). In humans, we have 23 MMPs with different but partially overlapping physiological roles, whereas in *Drosophila* only two MMPs are present and only one of them is expressed in the airway epithelium (Page-McCaw et al., 2003).

2.6 Potential Roles of Asthma Susceptibility Genes in *Drosophila*

One group of genes that is believed to comprise various highly asthma-associated members are the MMPs, mentioned above. As the genes MMP9 and MMP7 are considered as highly relevant for asthma (Table 2.1), its *Drosophila* homologue (DmMMP1) could be studied in order to tease apart its function independent of the other 21 MMPs (Goswami et al., 2009). Nevertheless, we have almost no experimentally based information regarding its direct role in asthma development and progression. In *Drosophila*, DmMMP1 has been shown to be important for tracheal development (Page-McCaw et al., 2003) and its expression is regulated upon airway infections (Isermann et al., personal communication). DmMMP1 as well as MMP9 are secreted and both accept collagen as a substrate, implying that they might act similarly. This suggests that a closer look at the physiological role of DmMMP1 in airway biology using the methodological armamentarium available in *Drosophila* will allow us a better understanding of MMP9's and MMP7's contribution to asthma pathology.

Apparently, *Drosophila* is a promising model for the study of the role of Toll receptors in asthma. Some Toll-like receptors such as TLR2, 4, 6, and 10 are asthma susceptibility genes. It has to be kept in mind that the central role of Toll receptors as pattern-recognition receptors of the innate immune system has been established in the fly (Lemaitre et al., 1996). *Drosophila* has nine different Toll receptors, but only one of them, Toll itself, has been shown to be relevant for immunity. In the airway epithelium, *Toll*, *18-wheeler*, *Toll-7*, and *Toll-8* are expressed (Wagner et al., 2008). Upon infection, Toll expression is increased in the airways (Wagner et al., 2009), which is puzzling as conventional Toll signaling is apparently not functional in this tissue (Wagner et al., 2008).

Another important group of asthma susceptibility genes comprise the signal transducer and activators of transcription (STATs). In the fly, the complexity of JAK/STAT signaling is reduced to its minimum. In mammals, multifarious families of ligands, receptors, JAK kinases and STATs are present, whereas in *Drosophila* only one receptor, one JAK kinase, and one STAT constitute this pathway (Arbouzova and Zeidler, 2006; Shuai and Liu, 2003). Since both, *STAT6* and

STAT3 are believed to play important roles in asthma pathogenesis, the presence of only one STAT homologue in the fly provides a unique opportunity to study the physiological significance of this factor in the airways. All components of the pathway, including the ligand *unpaired* (*upd*), are expressed in airway epithelial cells. In addition, we have shown that STAT-dependent signaling is operational in defined parts of the airways, without pathogen contact (Wagner et al., 2008). A more detailed analysis of JAK/STAT signaling in the airway epithelium of the fly has therefore great potential to provide new information about its role in health and disease of the airways.

Serpins (serine protease inhibitors) are also among those proteins that are relevant in asthma. One serpin, *SPINK5*, is a well-established asthma susceptibility gene. The copious number of genes with seemingly very similar functional roles complicates understanding the physiological and pathophysiological role of serpins. A functional equivalent in *Drosophila* is the serpin *Spn77Ba*. This serpin is present in the fly's airway epithelium and it plays an important role as a gatekeeper of the epithelial innate immune system. *Spn77Ba* disruption induces strong activation of the epithelial immune system but also of the systemic immunity. The latter response appears to be mediated by an unknown pathway, finally leading to release of anti-microbial peptides from the fat body, which is triggered by activation of the Toll pathway (Tang et al., 2008). These first results indicate that serpins might act as controllers of epithelial immunity, ensuring that immune-relevant proteases are inhibited in times of normal activities. Deregulation of their activity may thus induce prolonged inflammation and thereby contribute to asthma development.

Completely different types of molecules that are highly relevant in asthma are G-protein coupled receptors as major transducers of information. Adrenergic receptors are in a central position, because they are targeted by a huge number of asthma-related pharmaceuticals. With respect to this, most important may be the fly's orthologue of the adrenergic receptor, called *oct2ß*, which is expressed in the airway epithelium. It shares not only structural similarities but appears to mediate similar effects in the airways via similar second-messenger systems. The methods available to directly manipulate second-messenger systems in the airway epithelia can be used to study the effects of long-term medication with adrenergics, an aspect that is not understood at all.

We have a copious number of glutathione-S transferases (GST) at our disposal. Some of them are asthma susceptibility genes. GSTs are believed to act as general antioxidants that detoxify xenobiotics and facilitate normal lung function when their expression is increased in response to different types of stress. Unfortunately, we know little about their physiological role in the normal lung, and we also have limited experimental information about their significance in asthma development. However, all major GSTs known to be relevant for asthma pathogenesis have orthologues in the fly. These orthologues are expressed in the airways, allowing their functional analysis in the *Drosophila* model.

The full usefulness of the *Drosophila* model may emerge in combination with the most powerful genetic tools of today: genome-wide association (GWA) studies and second generation, whole genome sequencing. These new techniques promise that

discoveries of novel disease susceptibility loci, genes and disease-associated gene variants will reach unprecedented speed. As most of the genes located in susceptibility loci will be of unknown function and their involvement in asthma has not been documented, a quick but comprehensive test regarding their potential role in airway physiology and pathophysiology is absolutely mandatory. While genotyping and sequencing have made quantum leaps forward, functional genomics studies are still tedious, expensive, and time consuming. As such, there is the danger that we may overlook major parts of genome-wide association studies. Thus, translation of these results into basic asthma research and clinical applicability may be slowed down or hampered considerably at this stage.

Drosophila is a valuable tool to categorize putative candidate genes for further downstream analyses in vertebrate models. It may be used to dissect the fraction by which individual genes of a gene cluster contribute causally to an observed susceptibility signal. Even more importantly, it can be used to identify the relevance of a gene in a disease pathway or identify stimuli relevant for gene regulation. These potential applications are relevant for all complex diseases but especially for lung diseases such as asthma, because the organ of interest is difficult to access. Using *Drosophila* models, preliminary experiments with other species (especially with mice) may be reduced significantly, allowing a better focus of these time-consuming studies. Utilizing *Drosophila* in a systematic approach together with other models and tools seems most promising and may significantly reduce the turnaround time from genetic results into biologically meaningful data.

Taken together, *Drosophila* has the potential to develop into a complementary model in asthma research. The inherent strengths of this system can open a novel field in asthma research that might enable us to understand, at least in part, the role of important asthma susceptibility genes in detail.

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Chapter 3
Therapeutic Potential of Anti-Microbial Peptides from Insects

Jochen Wiesner and Andreas Vilcinskas

Abstract As an ancient mechanism of innate immunity virtually all multi-cellular organisms produce anti-microbial peptides (AMPs) that directly kill pathogens. Various AMPs, typically reaching high concentrations in the hemolymph, allow insects to cope with infections and to survive in habitats with massive microbial pressure. AMPs are also indispensable as part of the human innate immune system, besides the antibody- and T-cell-dependent adaptive immune system that exists only in vertebrates. Most AMPs act through permeabilization of the cell membrane of target organisms resulting in extremely rapid killing. Therefore, AMPs of various origins have been considered as lead structures for new anti-bacterial and anti-fungal drugs. While insect AMPs have only been tested in animal infection models so far, several artificial peptides derived from human, porcine, bovine, and frog AMPs have already undergone clinical trials. Here, an overview is provided of the different classes of AMPs found in insects, including a more detailed discussion of those peptides that have been proven to possess anti-microbial activity in experimentally infected animals. Further, the outcomes of the clinical studies on AMP-derived peptides are carefully reviewed. As will be seen, some peptides proved to be remarkably effective in the treatment of meningococcal sepsis, diabetic foot ulcers, acne, and catheter-related infections. However, no AMP-based drug has been approved so far. It finally will be discussed how exploiting the enormous repertoire of insect AMPs may lead to new drugs significantly improving today's armament of anti-microbial agents.

Keywords Anti-microbial peptides · Clinical studies · Drug development · Infectious diseases · Insect immune system

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3.1 The Insect Immune System

To defend themselves against pathogens, higher vertebrates have developed a sophisticated adaptive immune system. In mammals, adaptive immunity relies on distinct subsets of T and B lymphocytes responsible for various cell-mediated killing mechanisms together with production of different classes of antibodies. By virtue of rearrangeable V(D)J immunoglobulin gene segments, all jawed vertebrates, beginning with cartilaginous fish, generate a lymphocyte receptor and antibody repertoire of sufficient diversity to recognize the antigenic structures of virtually any potential pathogen. Lamprey and hagfish, which are the only surviving jawless fish belonging to the oldest vertebrate taxon, assemble diverse lymphocyte antigen receptor and soluble effector molecule genes through the rearrangement of leucine-rich repeat-encoding modules (Pancer and Cooper, 2006). Thus, recombinatorial mechanisms for the generation of anticipatory defense molecules evolved in both the jawless and jawed vertebrates, but each group employs a different kind of modular protein domain. Insects, like other invertebrates, lack an adaptive immune system. Nevertheless, many insects possess a powerful innate immune system that allows them to thrive in environments of enormous microbial pressure. Different types of hemocytes circulating in insects' hemolymph are involved in defense mechanisms such as phagocytosis, nodulation, encapsulation, melanization, and clotting (Strand, 2008). Since most studies were strictly morphological there is no clear terminology in naming insect hemocytes. The dominant professional phagocytes of *Drosophila* are called plasmatocytes, which are functionally homologous to the granulocytes in Lepidoptera. Phagocytosis is facilitated by a number of soluble and hemocyte membrane-bound pattern recognition receptors. Among the soluble receptors, functioning as opsonins, there are complement-like thioester-containing proteins (TEPs), C-type (calcium-dependent) lectins and other proteins binding to bacterial and fungal cell wall components. Some of the hemocyte surface receptors have direct mammalian homologues such as the Toll receptor, the peptidoglycan recognition protein, or the scavenger receptor SR-CI, whereas others appear to be unique to insects (Marmaras and Lampropoulou, 2009). Nodulation describes the formation of hemocyte aggregates around bacteria and fungi. Encapsulation occurs when hemocytes bind to larger structures such as nematodes or parasitoid eggs. Within the capsule the entrapped organisms are killed by the local production of reactive oxygen species, NO, or asphyxiation. Often, melanin is deposited within and around nodules and capsules. Melanin may form in response to lysis of crystal cells or oenocytoids in *Drosophila* or Lepidoptera, respectively, going along with the release of components of the prophenoloxidase (PO) cascade, in addition to activation of PO cascade components present in the plasma. Coagulation of hemolymph in response to external injury permits quick sealing of wounds, restoration of the hydrostatic skeleton, and entrapment of microbes at wound sites. Interaction of hemocytes with plasma factors is a general feature of coagulation in insects, although little is known about the molecular mechanisms, and considerable differences are found between species and within different life stages within species. In *Drosophila* larvae, coagulation is initiated when plasmatocytes are activated to degranulate by unknown

stimuli. Released clotting factors together with plasma factors lead to the formation of the primary soft clot. Additional hemocytes adhere to the clot. Subsequently, crystal cells are activated and rupture to release PO, which crosslinks and melanizes the clot to produce its mature form (Dushay, 2009). Besides cellular mechanisms, production of anti-microbial peptides (AMPs), exerting direct killing of microbes, represents the most important principle of insect immunity. In holometabolous insects, AMPs are mainly synthesized by the fat body from where they are released into the hemolymph (Bulet and Stöcklin, 2005). Most of these AMPs are produced quite massively after induction by septic injury, many of them reaching high micromolar concentrations. In heterometabolous insects, AMPs are constitutively produced and stored in hemocytes and released after immune challenge (Lamberty et al., 2001).

3.2 Classification of Anti-Microbial Peptides

3.2.1 Non-ribosomally Synthesized Peptides

To date, more than 1,000 peptides and proteins with anti-microbial activity derived from virtually all species of life, from bacteria to humans, have been described. These molecules principally fall into two major classes, ribosomally and non-ribosomally synthesized peptides. While the former are found in all groups of organisms, the latter are typically produced by bacteria and fungi. Non-ribosomally synthesized peptides are assembled by multi-enzyme complexes and typically contain D-amino acids and other non-proteinogenic amino acids, often have a cyclic or branched structure and may carry various modifications. By this definition, many commonly used antibiotics are peptide derived (Hancock and Chapple, 1999). The natural penicillins and cephalosporin C, for example, can be dissected into several amino acids. Larger non-ribosomally synthesized peptide antibiotics in current clinical use are represented by bacitracin, gramicidin S, polymyxin B, the streptogramins, and the glycopeptide class of antibiotics, including vancomycin and teicoplanin.

3.2.2 Bacteriocins

The ribosomally synthesized anti-microbial peptides and proteinaceous defense molecules of bacteria are collectively called bacteriocins and can be divided into those from Gram-positive and Gram-negative bacteria (Gillor et al., 2008). Lantibiotics represent a group of bacteriocins from Gram-positives characterized by the unusual amino acid lanthionine, that is produced by the post-translational introduction of thioether bridges (Piper et al., 2009). The best known lantibiotic, nisin, is routinely used by the food industry as a preservative. The bacteriocins from Gram-negative bacteria are mainly represented by colicins, microcins, and pyocins.

Colicins are a family of high molecular weight (25–80 kDa) anti-microbial proteins produced by *Escherichia coli* with narrow host ranges acting primarily on other strains of *E. coli* and its close enteric relatives (Holtsmark et al., 2008). A colicin protein is composed of three domains, one involved in recognition of specific cell surface receptors, one mediating translocation through the cell envelope, and one exerting the killing activity by either pore-formation, inhibition of cell wall synthesis, or degradation of nucleic acids. Microcins form a very restricted group of bacteriocins derived from *E. coli* and other enteric bacteria. Only 14 microcins have been reported so far, seven of which have been fully characterized (Gillor et al., 2008). Typically, microcins rely on a "Trojan horse" strategy by mimicking essential molecules which are taken up by susceptible Gram-negative bacteria via specific receptors. The actual killing is mediated by diverse mechanisms such as pore formation, membrane destabilization as well as inhibition of DNA, RNA and protein synthesis (Duquesne et al., 2007). Two different types of microcins may be distinguished, the first representing small peptides (<3 kDa) that are generally highly modified and the second polypeptides between 7 and 10 kDa that can be modified or not. Pyocins are produced by most *Pseudomonas aeruginosa* strains (Michel-Briand and Baysse, 2002). Three types of pyocins are described. R-type pyocins resemble non-flexible, contractile tails of bacteriophages. F-type pyocins also resemble phage tails, but with a flexible, non-contractile structure. S-type pyocins are soluble colicin-like proteins. Pyocins primarily kill other *P. aeruginosa* strains, however, R-type pyocins are also active against other Gram-negative bacteria. Entities similar to the R-type pyocins are also present in other Gram-negative bacteria and even in the Gram-positive organisms *Listeria monocytogenes* and *Staphylococcus aureus* (Scholl and Martin, 2008). Bacteriocin-like molecules from archea have been identified in several species of the family *Halobacteriaceae* and in the hyperthermophile *Sulfolobus islandicus* (Prangishvili et al., 2000; O'Connor and Shand 2002).

3.2.3 Anti-Microbial Peptides of Multi-Cellular Organisms

The term "anti-microbial peptides" in a more restricted sense is used for peptides from multi-cellular organisms with anti-bacterial, anti-fungal, anti-parasitic and/or anti-viral activity. Most AMPs are composed of 12–50 amino acid residues and differ from most bacteriocins in several respects. Bacteriocins are typically highly active at very low concentrations but target only a very restricted spectrum of susceptible organisms. In addition, their structures are often quite unique reflecting very distinct mechanisms of killing. In contrast, AMPs from multi-cellular organisms are less specific, inhibiting the growth of a broader range of pathogens at comparably high concentrations. Despite low similarity at the amino acid sequence level the great majority of AMPs fall within one of the three structural classes: (1) linear alpha-helical peptides free of cysteine residues, (2) peptides adopting a beta-sheet globular structure stabilized by intra-molecular disulfide bridges, (3) peptides with unusual bias in certain amino acids, such as proline, glycine, arginine,

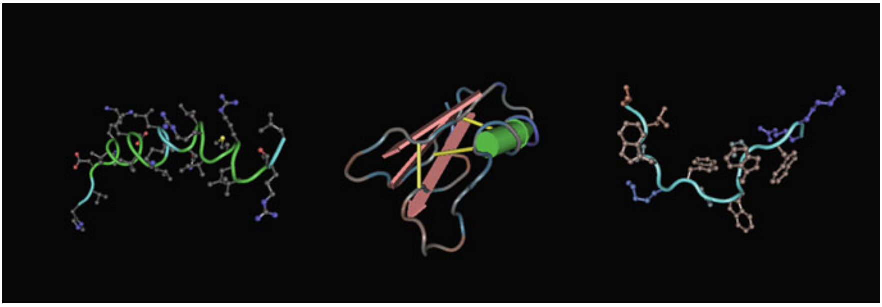


Fig. 3.1 Examples for the three basic types of AMPs in multi-cellular organisms. *Left*: The α-helical spinigerin from the termite *Pseudacanthotermes spiniger* (MMDB ID: 35132). *Middle*: Heliomicin from the lepidopteran *Heliothis virescens* composed of three β-strands and one α-helix; the three disulfide bonds stabilizing the tertiary structure are indicated in *yellow* (MMDB ID: 18720). *Right*: Bovine indolicidin with an unusually high content of tryptophan residues (MMDB ID: 73620)

tryptophan, or histidine (Boman, 2003) (Fig. 3.1). AMPs with very similar structural patterns belonging to one of the three classes are found in widely different organisms. Therefore, classification of AMPs on a taxonomical basis is often found to be less appropriate (Andreu and Rivas, 1998).

3.3 Mode of Action

Although the exact killing mechanism of different AMPs may be quite different in detail, permeabilization of membranes is a repeating theme. Most of these peptides possess a net positive charge and consist of approximately 50% hydrophobic amino acid residues. Primary binding of most AMPs to target membranes is mediated by electrostatic interactions. The outer leaflet of mammalian cells is almost exclusively composed of zwitterionic or uncharged lipids, mainly phosphatidylcholine, sphingomyelin, glycosphingolipids, and cholesterol, whereas bacterial membranes contain large amounts of negatively charged lipids such as phosphatidylglycerol and cardiolipin (Matsuzaki, 1999). In addition, the outer membrane of Gram-negative bacteria is covered with polyanionic lipopolysaccharides, and the cell wall of Gram-positives carries negatively charged teichoic acids. Consequently, the bacterial surface is highly negatively charged whereas the outside of mammalian cells is typically uncharged, conferring selectivity to the action of AMPs. In the aqueous phase, many AMPs are essentially unstructured and fold upon contact with the target membrane, adopting an amphiphilic conformation (Giuliani et al., 2008). Adsorption of the peptides onto the lipid bilayer and their subsequent integration into the membrane results in expansion of the outer leaflet, which in turn leads to membrane thinning. Since the latter effect is not uniformly distributed over the

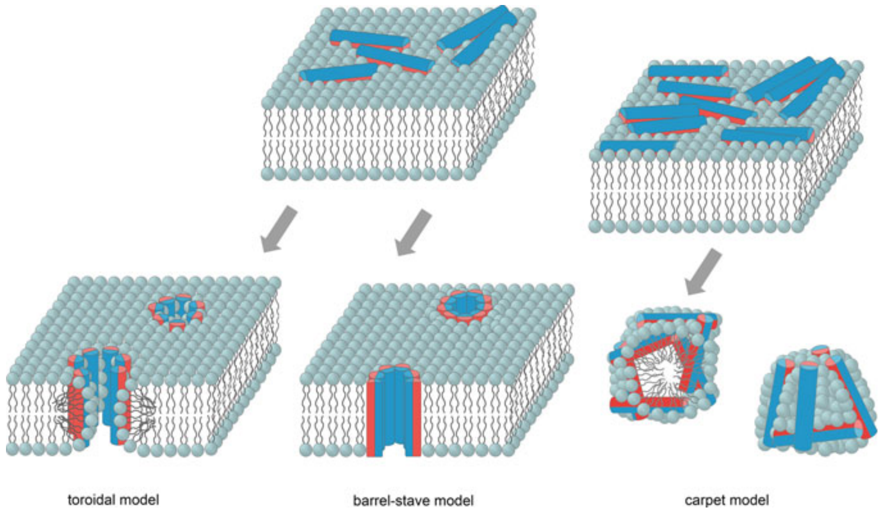


Fig. 3.2 Three different models for pore formation and the disruption of lipid bilayers by amphiphilic anti-microbial peptides. According to the toroidal model, the peptides aggregate and force the lipid monolayers to bend continuously through the pore in a way that both the peptides and the lipid head groups line the aqueous core. In the barrel-stave model, hydrophobic peptide regions align with the lipid core region and the hydrophilic peptide regions form the inner surface of the pore channel. The carpet model assumes that a large number of peptides orientate in parallel to the membrane surface. At high peptide concentrations the membrane is disrupted in a detergent-like manner by the formation of micelles

entire bilayer area, transient pores are formed over a certain concentration threshold. Several models have been proposed for pore formation such as the toroidal (or worm-hole), barrel-stave, or carpet-like model (Brogden, 2005) (Fig. 3.2). AMPs with anti-cancer activity apparently bind to negatively charged gangliosides present in high concentration on tumor cells (Lee et al., 2008). In contrast, binding of some AMPs to fungal membranes is charge-independent with neutral glucosylceramides playing a central role in anti-fungal action (Aerts et al., 2008). Formation of ion channels, trans-membrane pores and extensive membrane rupture leads to the dissipation of trans-membrane electrochemical ion gradients, loss of metabolites, and eventually to lysis of microbial cells. However, some AMPs such as buforin I from frogs (Lee et al., 2008), the pea defensin Psd1 (Lobo et al., 2007), and the short-chain proline-rich insect AMPs are known to translocate into the cytoplasm without permeabilizing the cytoplasmatic membrane. While buforin I binds to nucleic acids and Psd1 apparently interferes with proteins involved in cell cycle control, inhibition of a chaperon was demonstrated for the short-chain proline-rich AMPs, and there is increasing evidence that a huge number of AMPs also address cytoplasmatic targets in addition to the cell membrane. Such intra-cellular targets include inhibition of DNA, RNA and protein synthesis, inhibition of cell-wall synthesis, activation of autolysins, inhibition of various enzymatic activities, and induction of apoptotic pathways (Brogden, 2005).

3.4 Classes of Insect AMPs

3.4.1 α-Helical AMPs

Cecropins together with sarcotoxins, hyphancin, enbocin, and spodopsin and other cecropin-like peptides represent the most abundant family of linear α-helical AMPs in insects. Cecropins A and B isolated from the hemolymph of the giant silk moth *Hyalophora cecropia* were the first inducible animal AMPs fully characterized (Steiner et al., 1981). In contrast to the previously known melittin, an α-helical peptide from bee venom with some anti-microbial properties, the cecropins did not display lytic activity against mammalian cells. Additional cecropin-like AMPs were identified in various other insects, which all belong to phylogenetically higher orders of Diptera and Lepidoptera (Bulet and Stöcklin, 2005). Dipteran cecropins form a distinct group of peptides with a high degree of amino acid sequence similarity. Perfect sequence identity was found between cecropin IA (also named sarcotoxin IA) from the flesh fly *Sarcophaga peregrina* and cecropin A from *Drosophila melanogaster* (Kylsten et al., 1990). In contrast, cecropins from Lepidoptera have greater variance. Typically, cecropins contain a tryptophan residue in position 1 or 2 and are amidated at the C-terminus (Fig. 3.3). However, both characteristics may be absent as in the mosquito cecropins from *Aedes albopictus* and *A. gambiae* (Lowenberger et al., 1999). In a hydrophobic environment cecropins adopt a rather stable conformation characterized by a long N-terminal, basic, amphiphilic α-helix followed by a shorter and more hydrophobic C-terminal helix. Other types of α-helical AMPs have been characterized such as ceratotoxin from the medfly *Ceratitis capitata*, stomonxyn from the stable fly *Stomoxys calcitrans* and spinigerin from the termite *Pseudacanthotermes spiniger* (Bulet et al., 2004). A cecropin-like secondary structure of stomoxyn was solved by CD and NMR spectroscopy, while spinigerin was found to form a well-defined slightly bent amphiphilic α-helix with the hydrophobic face situated on the concave side, very similar to frog magainin 2 (Landon et al., 2006). In contrast to cecropins, which are primarily active against Gram-negative bacteria, stomoxyn was shown to kill Gram-negatives, Gram-positives, filamentous fungi, yeast, and *Trypanosoma brucei rhodesiense*, the causative agent of African sleeping sickness transmitted by tsetse flies. As *S. calcitrans* is sympatric with tsetse flies and feeds on many of the same vertebrate hosts, this finding was suggested to explain, at least in part, why *S. calcitrans* is not a vector of African trypanosomiasis (Boulanger et al., 2002).

The in vivo efficacy of cecropin A and the frog AMP magainin 2 was tested in rats experimentally infected with *P. aeruginosa* (Cirioni et al., 2008). The animals were intra-peritoneally inoculated with either a *P. aeruginosa* reference strain or a multi-drug-resistant clinical isolate. For treatment, 1 mg/kg cecropin A or 1 mg/kg magainin 2 were administered by intra-venous injection immediately or 6 h after infection. In parallel, groups of animals received rifampicin (10 mg/kg), tazobactam/piperacillin (120 mg/kg), or rifampicin combined with either of the two AMPs. In control animals receiving saline only, the lethality of the challenge was 100% within 72 h. Lethality of the animals treated with either of the two AMPs ranged

Hyalophora cecropia	Cecropin A	KW--KLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQI---AK*
Sarcophaga peregrina	Sarcotoxin IA	GWLKKIGKKIERVGQHTRDATIQ-GLGIA--QQAANVAATAR*
Aedes aegypti	Cecropin A	GGLKKLGKKLEGAGKRVFNAAEKA---LPVVAGAKALR---K
Ceratitis capitata	Ceratotoxin	SIGSAF-KKALPVAKKIGKAALPIAKAALP
Stomoxys calcitrans	Stomoxyn	RGFRKHFNKLVKKVKHTISETAHVAKDTAVIAGSGAAVVAAT*
Pseudacanthotermes spiniger	Spinigerin	HVDKKVADKVLLLKQLRIMRLLTRL
Apis mellifera	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ*

Fig. 3.3 Primary structures of selected α-helical insect AMPs. The *asterisk* (∗) marks C-terminal amidation

Fig. 3.4 Short hybrid AMPs derived from cecropin A and melittin

CA1-7/M2-9	KWKLFKK	IGAVLKVL
CA1-7/M5-9	KWKLFKK	VLKVL
CA1-8/M6-9	KWKLFKKI	LKVL
CM4	**WK**KLFKK	LK**I**L

between 47 and 67% compared to 40–67% for the tazobactam/piperacillin treatment. Only very slight reduction of lethality was seen with rifampicin (87–100% lethality). The combination of the AMPs with rifampicin resulted in improved efficacy (27–33% lethality). There was a trend towards slightly higher activity of magainin compared to cecropin A, and reduced efficacy of all treatments against the multi-drug-resistant strain. However, these differences appeared to be within the experimental variations.

Early efforts to come up with AMPs of substantially reduced length resulted in the synthesis of various cecropin A-melittin hybrids such as the 15-residue CA1-7/M2-9 (Andreu et al., 1992) (Fig. 3.4). The anti-bacterial activity of CA1-7/M2-9 was essentially comparable to the parent peptides. Furthermore, CA1-7/M2-9 was active against *S. aureus*, including methicillin-resistant nosocomial isolates, and blood stages of the malaria parasite *Plasmodium faciparum*, while cecropin A is inactive against these pathogens (Andreu et al., 1992; Giacometti et al., 2004). CA1-7/M2-9 and related hybrid peptides proved also to be active against other Gram positives and anaerobic species of clinical relevance (Oh et al., 2000). Most important, CA1-7/M2-9 was devoid of hemolytic activity characteristic for full-length melittin. In order to improve protease resistance, the *all*-D enantiomers of six 15-residue cecropin A-mellitin hybrids were synthesized (Merrifield et al., 1995). As expected, the D enantiomers were resistant against trypsin, while the L enantiomers were rapidly degraded. In each case, the D and L enantiomers displayed equal anti-microbial activity within experimental error, pointing to the absence of chiral targets such as enzymes or proteinaceous receptors.

CA1-7/M2-9 together with four additional cecropin A-melittin hybrids ranging from 12 to 18 residues was tested in a keratitis model in rabbits (Nos-Barbera et al., 1997). Bacteria of a *P. aeruginosa* reference strain were inoculated into the stroma of one cornea of each rabbit. The peptides were applied as 0.1% solution, 30 μl to each eye, six dosages every 2 h. In a separate experiment, a clinical isolate of *P. aeruginosa* was used for infection, and the treatment carried out by 12 instillations, applied hourly. In both experiments, control animals were treated with 0.3% gentamycin eye drops. The animals were killed at 24 h for ethical reasons after it had

become evident that all treatments resulted in an inhibition of the induced pathology with statistical significance, compared to vehicle-treated rabbits. All peptides proved to be equally potent with efficacy comparable to gentamycin. In the first experiment there was a non-significant trend to higher efficacy of gentamycin. Remarkably, no loss of activity was observed with the shortest peptide (CA1-7/M5-9) consisting of only 12 residues (Fig. 3.4).

Short cecropin A-melittin hybrid peptides were also shown to possess remarkable in vitro activity against the promastigote forms of the protozoan parasite *Leishmania donovani* (Luque-Ortega et al., 2003). The 12-residue CA1-8/M6-9 (Fig. 3.4) displayed equal or slightly superior activity compared to the 26-residue CA1-8/M1-18. Similar activity was found with the 11-residue CM4 (Fig. 3.4) characterized by three amino acid substitutions compared to cecropin A and melittin. Another study attempted to improve the leishmanicidal activity of CA1-7/M2-9 by the attachment of fatty acid residues (Chicharro et al., 2001). This type of modification increased the activity up to threefold against promastigotes and up to 15-fold against amastigotes. The usefulness of these derivatives was somewhat compromised by their tendency to aggregate and to adhere to surfaces. One of these derivatives (Oct-CA1-7/M2-9), obtained by the attachment of octanoic acid to the α amino group of the N-terminal lysine residue was tested for the treatment of dogs with naturally acquired *Leishmania infantum* infection (Alberola et al., 2004). Eight dogs with a recent diagnosis of canine leishmaniasis were treated by intra-venous injection of 5 mg of the peptide at day 0, 2, and 4. One week after the last dose, the dogs were treated with the standard antimony therapy because leishmaniasis is a life-threatening disease and there was no previous knowledge about the efficacy of the study drug. In response to the treatment, improvement of the general health status and a progressive diminution in parasitemia was reported. No changes in laboratory parameters were found, probably due to the short follow-up period, since normalization may take months.

3.4.2 Disulfide-Stabilized AMPs

AMPs stabilized by intra-molecular disulfide bonds are widely distributed in insects. Typically they contain three disulfide bonds, but also peptides with four (drosomycin) or only one (thanatin) disulfide bond are known (Fig. 3.5). Those with three or four disulfide bonds are commonly referred to as insect defensins because of their over-all structural similarities to mammalian α- and β-defensins (Bulet and Stöcklin, 2005). Insect defensins can be subdivided in peptides with an α-helix/β-sheet (αββ, βαββ) mixed structure and peptides forming triple-stranded anti-parallel β-sheets (βββ).

Insect defensins are generally larger than cecropins and may carry N-terminal (*S. calcitrans* defensin) or C-terminal (royalisin from royal jelly) extensions. With some exceptions such as royalisin and termcidin, insect defensins are typically not amidated. The first insect defensins were isolated from cultured *S. peregrina* cells and experimentally injured larvae of the black blowfly (*Proto*) *phormia terraenovae*

AMPs with a single disulfide bridge		
P. maculiventris	Thanatin	GSKKPVPIIY**C**NRRTGK**C**QRM
AMPs with three disulfide bridges		
S. peregrina	Sapecin	AT**C**DLLSGTGINHSA**C**AAH**C**LLRGNRGGY**C**NGKAV**C**V**C**RN
P. terraenovae	Defensin A	AT**C**DLLSGTGINHSA**C**AAH**C**LLRGNRGGY**C**NGKGV**C**V**C**RN
S. calcitrans	Defensin	AAKPMGIT**C**DLLSLWKVGHAA**C**AAH**C**LVLGDVGGY**C**TKEGL**C**V**C**KE
A. mellifera	Royalisin	VT**C**DLLSFKGQVNDSA**C**AAN**C**LSLGKAGGH**C**EKGV**C**I**C**RKTSFKDLWDKRF*
A. cyanea	Defensin	GFG**C**PLDQMQ**C**HRH**C**QTITGRSGGY**C**SGPLKLT**C**T**C**YR
G. mellonella	Gallerimycin	GVTITVKPPFPG**C**VFYE**C**IAN**C**RSRGYKNGGY**C**TING**C**Q**C**LR
H. virescens	Heliomicin	DKLIGS**C**VWGAVNYTSD**C**NGE**C**KRRGYKGGH**C**GSFANVN**C**W**C**ET
P. spiniger	Termicin	A**C**NFQS**C**WAT**C**QAQHSIYFRRAF**C**DRSQ**C**K**C**VFVRG*
A. longimanus	Alo3	**C**IKNGNG**C**QPNGSQGN**CC**SGY**C**HKQPGWVAGY**C**RRK
AMPs with four disulfide bridges		
D. melanogaster	Drosomycin	D**C**LSGRYKGP**C**AVWDNET**C**RRV**C**KEEGRSSGH**C**SPSLK**C**W**C**EG**C**

Fig. 3.5 Primary structures of selected insect AMPs stabilized by disulfide bridges. Cysteine residues participating in the disulfide bonds are in *bold face*

(Matsuyama and Natori, 1988; Lambert et al., 1989). Various further defensins were identified in insects belonging to the phylogenetically recent orders Diptera, Lepidoptera, Coleoptera, Hymenoptera, and to the ancient order of Odonata (dragonflies). Sequence comparison revealed 70 to almost 100% amino acid similarity among dipteran defensins and indicated that these defensins have more than 35% similarity to the defensin of the dragonfly *Aeschna cyanea* (Bulet et al., 1999). Remarkably, the dragonfly defensin is more closely related to mollusk and scorpion defensins than to the dipteran defensins. Most insect defensins described so far exert primarily anti-bacterial activity and are particularly active against Gram-positive bacteria. A smaller number was found to be predominantly or strictly antifungal, with pronounced activity against filamentous fungi. Anti-fungal defensins include gallerimycin from the greater wax moth *Galleria mellonella*, heliomicin from the tobacco budworm *Heliothis virescens*, termicin from the termite *P. spiniger*, Alo3 from the harlequin beetle *Acrocinus longimanus*, and drosomycin from *D. melanogaster*. The anti-bacterial defensins and termicin adopt the αββ structure, while an additional β-strand (βαββ) is present in heliomicin and drosomycin. Drosomycin exhibits considerable similarity to plant defensins, that are characterized by four disulfide bonds, the βαββ pattern, and mainly anti-fungal activity (Wong et al., 2007). The typical and unique βββ structure has been observed for Alo3 (Barbault et al., 2003).

Because of their highly compact structure resulting in remarkable protease resistance, insect defensins are considered to represent valuable templates for new anti-infective agents. Particular interest has been attracted by heliomicin as it is active against several fungi responsible for severe nosocomial infections and retains activity at physiological ionic strength. Screening for natural analogues of heliomicin led to the isolation of a peptide called ARD1 (Fig. 3.6) from the hemolymph of immuno-stimulated *Archaeoprepona demophoon* caterpillars (Landon et al., 2004). Despite only two amino acid changes compared to heliomicin, ARD1 proved to possess approximately twofold increased anti-fungal activity. Further improvement of activity was achieved by the recombinant production of ARD1 analogues with

Heliomicin	DKLIGSCVWGAVNYTSDCNGECKRRGYKGGHCGSFANVNCWCET
ARD1	DKLIGSCVWGAVNYTS**N**CN**A**ECKRRGYKGGHCGSFANVNCWCET
ETD151	DKLIGSCVWGAVNYTS**N**C**RA**ECKRRGYKGGHCGSFANVNCWCET

Fig. 3.6 Primary structures of the anti-fungal peptide heliomicin of *Heliothis virescens* and the related peptide ARD1 of *Archaeoprepona demophoon*. ETD151 represents an artificial homologue of ARD1

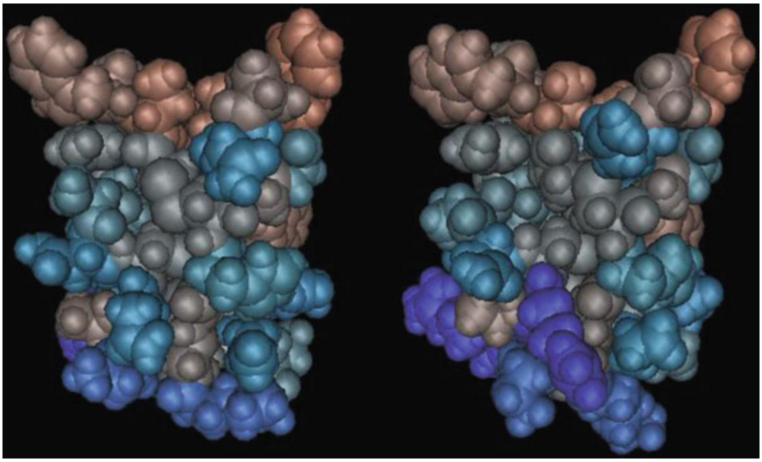


Fig. 3.7 Structures of the anti-fungal peptide ARD1 from *Archaeoprepona demophoon* (*left*) and its synthetic derivative ETD151 (*right*). The single amino acid exchange (asparagine to arginine) results in markedly increased amphiphilicity. Hydrophobic and hydrophilic amino acid residues are labelled in brown and blue respectively (MMDB IDs: 26812 and 26813)

various single amino acid substitutions aiming to increase cationicity, hydrophobicity or both. The most successful peptide termed ETD151 (Fig. 3.6) with at least twofold increased activity compared to ARD1 resulted from an asparagine to arginine exchange. The direct impact of the modification on the amphipathic properties of the molecule was demonstrated by 3D NMR structure analysis (Fig. 3.7). In preliminary pre-clinical studies, ETD151 was administered to animals by continuous intra-venous infusion at doses up to 600 mg/kg with no signs of toxicity (Dimarcq and Hunneyball, 2003).

Thanatin isolated from the spined soldier bug *Podisus maculiventris* contains only two cysteine residues, forming a single intra-molecular disulfide bridge (Fehlbaum et al., 1996). Remarkable structural similarities are found between thanatin, brevinins from frog skin and the non-ribosomally synthesized peptide antibiotic polymyxin, as these peptides are characterized by a flexible N-terminal arm attached to a compact octapeptide (thanatin) or heptapeptide (brevinins and polymyxin) ring at the C-terminus (Clark et al., 1994; Mandard et al., 1998) (Fig. 3.8). Thanatin has broad-spectrum activity killing both Gram-positive and Gram-negative bacteria, filamentous fungi, but apparently no yeast, with no conceivable hemolytic activity (Fehlbaum et al., 1996; Wu et al., 2008). The *all*-D

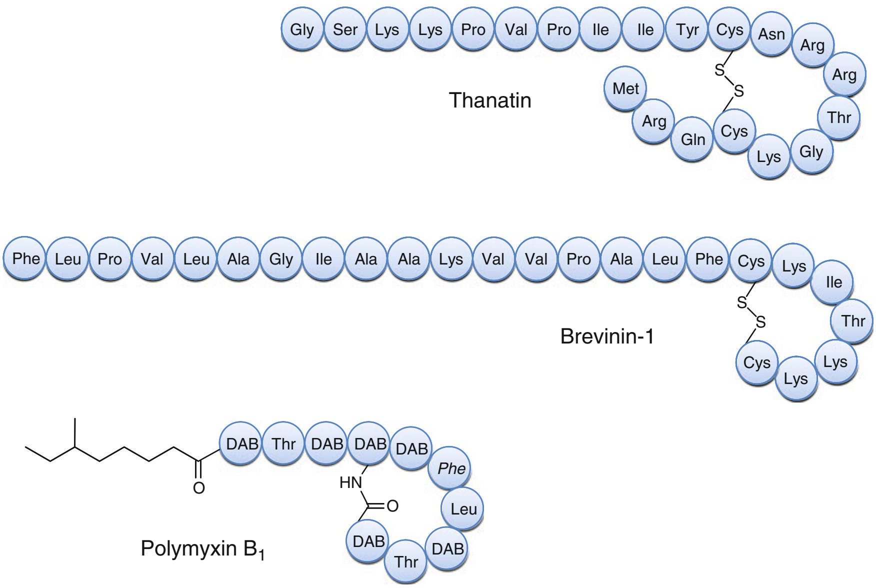


Fig. 3.8 Schematic presentations of insect thanatin, frog brevinin-1, and the non-ribosomally synthesized peptide antibiotic polymyxin B_1. Polymyxin B_1 contains the non-proteinogenic amino acids L-α,γ-diaminobutyric acid (DAB) and D-phenylalanine (*italic*)

enantiomer of thanatin retained full activity against fungi, but was inactive against Gram-negative bacteria (Fehlbaum et al., 1996). The activity of *all*-D thanatin against Gram-positives appeared to be species-dependent. This suggests that thanatin acts through different mechanisms, involving chiral targets in Gram-negative bacteria but not in fungi and some Gram-positive bacteria. Alkylation of the two cysteine residues of thanatin, preventing the formation of the disulfide bond, decreased the activity against the Gram-negative bacterium *E. coli*, while the activity against *Micrococcus luteus*, a Gram-positive organism, was improved (Orikasa et al., 2009). It was found that the activity against *M. luteus* increased with the hydrophobicity of the alkyl chain, with the octyl derivative being eight times more active than native thanatin. Reportedly, a thanatin derivative with the threonine residue substituted by serine (S-thanatin) could reduce the mortality of animals with experimental peritonitis induced by cercal ligation and puncture (Wu et al., 2009).

3.4.3 Proline-Rich AMPs

AMPs with a bias for certain amino acids are represented in insects mainly by proline-rich peptides. In addition, groups of comparably large anti-microbial polypeptides with an over-representation of glycine residues are found. Proline-rich AMPs have been isolated from Hymenoptera (apidaecins, abaecins, and formaecins), Diptera (drosocin, metchnikowin), Hemiptera (pyrrhocoricin, metalnikowins), and Lepidoptera (lebocins). The size of the proline-rich AMPs varies

Short-chain proline-rich AMPs		
Apis mellifera	Apidaecin Ia	GN--NRPVYIPQPRPP-HPR-I
	Apidaecin Ib	GN--NRPVYIPQPRPP-HPR-L
Bombus pascuorum	Apidaecin	GN---RPVYIPPPRPP-HPR-L
Myrmecia gulosa	Formaecin-1	G-RPN-P-VNNK--P**T**PHPR-L
	Formaecin-2	G-RPN-P-VNTK--P**T**PYPR-L
D. melanogaster	Drosocin	G-KP-RP-Y-**S**-PRP**T**SHPRPI--RV
Pyrrhocoris apterus	Pyrrhocoricin	VDKG---SYL--PRP**T**-PPRPIYNRN
Palomena prasina	Metalnikowin-1	VDKP---DYR--PRPR-PPNM
	Metalnikowin-2A	VDKP---DYR--PRPW--PRPN
	Metalnikowin-2B	VDKP---DYR--PRPW--PRNMI
	Metalnikowin-3	VDKP---DYR--PRPW--PRPNM
Long-chain proline-rich AMPs		
Bombyx mori	Lebocin-1 and -2	DLRFLYPRGKLPVP**T**P---P--PFNPKPIYIDMGNRY
	Lebocin-3	DLRFLYPRGKLPVP**T**L---P--PFNPKPIYIDMGNRY
Apis mellifera	Abaecin	YVPLPNVPQPGRRPFPTFPGQ--GPFNPK-IKWPQG--Y
Bombus pascuorum	Abaecin	FVPY-NPPRPGQ-SKPFPSFPGHGPFNPK-IQWP----YPLPNPGH
D. melanogaster	Metchnikowin-1	HRHQGPIFDTRPS-PFNPNQP-RP-GPIY
	Metchnikowin-2	HRRQGPIFDTRPS-PFNPNQP-RP-GPIY

Fig. 3.9 Examples of proline-rich insect AMPs. *O*-glycosylated residues (*bold face*) can carry *N*-acetylgalactosamine or galactose → *N*-acetylgalactosamine side chains. Lebocin-1 and -2 differ by modification with the disaccharide and monosaccharide, respectively

from 15 to 39 residues, with metalnikowin-1 and -2A from *Palomena prasina* and abaecin from the bumblebee *Bombus pascuorum* as examples for the two extremes. Short-chain (<20 residues) and long-chain (>20 residues) proline-rich AMPs may be distinguished as two sub-families (Fig. 3.9). The proline residues are typically associated in doublets or triplets with basic residues, most frequently with arginine. The characteristic PRP or PHP motif occurs in all known short-chain and most but not all long-chain proline-rich AMPs. On some of the proline-rich AMPs, *O*-linked carbohydrates are attached to conserved threonine residues (Bulet et al., 1993; Cociancich et al., 1994; Hara and Yamakawa, 1995; Mackintosh et al., 1998). Lebocin-2 and -3, pyrrhocoricin, and formaecin-1 and -2 are modified by an *N*-acetylgalactosamine monosaccharide (GalNAcα1-Thr) while the modification of lebocin-1 and drosocin is an *N*-acetylgalactosamine-galactose disaccharide (Galβ1-3GalNAcα1-Thr). There is also one report on drosocin being glycosylated on Ser 7 in addition to Thr 11 (Rabel et al., 2004). The biological role of the glycosylation remains unclear as the presence of mono- and disaccharide side chains improves the activity of drosocin and formaecin 1, but synthetic glycan-free pyrrhocoricin is more active than the natural glycosylated peptide (Bulet et al., 1996; Hoffmann et al., 1999). Among the lebocins, the derivatives without glycosylation are less active than the natural peptides with a monosaccharide side chain. However lebocin-1, which carries a disaccharide, is somewhat less active than the corresponding lebocin-2 carrying a monosaccharide (Hara and Yamakawa, 1995). NMR structure analysis revealed that in a membrane-mimicking solvent drosocin mainly exists as random coils with a small population of folded conformers characterized by turn elements preferentially near residues 4–7, 10–13, 17 and 18 (McManus et al., 1999). No substantial differences between the glycosylated and non-glycosylated forms were observed in the random coil conformation, but upon glycosylation at Thr 11 a slight

straightening of the turn at residues 10–13 going along with some tightening of the turn at residues 17 and 18 was seen in the small subpopulations of folded peptides.

The anti-microbial activity of the short-chain proline-rich AMPs is mainly directed against Gram-negative bacteria, while the peptides of the long-chain type appear to possess more broad-spectrum activity (Otvos, 2002). Metchnikowin was found to be inactive against *E. coli* (Gram-negative) but killed *M. luteus* (Gram-positive). In addition, metchnikowin displayed activity against *Neurospora crassa*, *Fusarium graminearum*, and *Blumeria graminis*, filamentous fungi of the phylum Ascomycota, the latter two species representing important plant pathogens. In contrast, no activity was observed against *Rhizoctonia solani* and *Piriformospora indica*, belonging to the Basidiomycota (Levashina et al., 1995; Rahnamaeian et al., 2009).

Unlike most other AMPs, which kill bacteria within minutes in a non-stereospecific fashion, the short-chain proline-rich AMPs need hours and only affect actively growing cells. No membrane permeabilization was observed with apidaecin at concentrations exceeding bactericidal doses by four orders of magnitude, and apidaecin-resistant mutants remained fully susceptible to pore-forming peptides (Casteels and Tempst, 1994). In addition, *all*-D enantiomers of apidaecins, drosocin, and pyrrhocoricin are totally inactive. It is supposed that the mode of action of short-chain proline-rich AMPs ultimately depends on inactivation of bacterial DnaK, a member of the highly conserved heat shock protein family (Hsp70) (Kragol et al., 2001). DnaK is a chaperon that interacts with short hydrophobic segments of non-native protein conformers generated in multiple cellular processes such as protein synthesis, protein translocation, protein re-folding, and assembly and disassembly of protein complexes. Pyrrhocoricin was shown to bind to the hydrophobic substrate-binding site of DnaK, which functions as a lid folding over the active site. Thus, pyrrhocoricin prevents access of polypeptidic substrates to the binding pocket and, as a consequence, inhibits ATPase activity which resides in a second domain of DnaK (Morell et al., 2008; Liebscher and Roujeinikova, 2009). Pyrrhocoricin fails to bind to mammalian Hsp70 homologues, and it was shown that drosocin-susceptible bacteria share a highly homologous amino acid sequence in the lid region of DnaK. *M. luteus* and *Xanthomonas campestris* appear to be the exception to the rule and may be killed by unrelated mechanisms (Bikker et al., 2006). Studies with synthetic peptide fragments of pyrrhocoricin demonstrated that the DnaK inhibitory activity is associated with the N-terminal half, while translocation through the cell membrane is mediated by the C-terminal half. Translocation appeared to be quite unspecific as fluorescein-labelled peptides were found to be able to enter not only susceptible Gram-negative bacteria cells but also Gram-positive bacteria cells, some mammalian cells and cells of the protozoan parasite *Cryptosporidium parvum* (Kragol et al., 2002; Otvos et al., 2004; Boxell et al., 2008). The limited toxicity for mammalian cells can be explained by the lack of an appropriate intra-cellular DnaK/Hsp70 target.

Initial experiments to treat *E. coli* infected mice with drosocin were unsuccessful. This was explained by the low stability of the peptide in mammalian blood compared to insect hemolymph (Hoffmann et al., 1999). Pyrrhocoricin was subsequently

Pyrrhocoricin	VDKGSYLPRPTPPRPIYNRN
Chex-pyrrhocoricin-Dap(Ac)	ChexDKGSYLPRPTPPRPIYNRDap(Ac)
Chex1-Arg20	ChexRPDKPRPYLPRPRPPRPVR

Fig. 3.10 Pyrrhocoricin and two synthetic derivatives with proven in vivo anti-bacterial activity. Chex, 1-amino-cyclohexane-carboxylic acid; Dap(Ac), β-acetyl-diamino-propionic acid

found to be more stable in human and mouse serum, albeit considerable degradation still occurred (Otvos et al., 2000). Mice infected with *E. coli* were protected by pyrrhocoricin administered intravenously at doses of 10 and 25 mg/kg at 1 and 5 h after challenge. However, toxicity was observed at 50 mg/kg. In order to prevent exopeptidase cleavage, a pyrrhocoricin analogue was synthesized in which Val 1 was replaced with 1-amino-cyclohexane-carboxylic acid and Asn 20 with β-acetyl-diamino-propionic acid (Fig. 3.10). This new entity [Chex-pyrrhocoricin-Dap(Ac)] proved to have similar activity to pyrrhocoricin in the *E. coli* mouse infection model, but without signs of toxicity at the 50 mg/kg dose level.

In further studies, artificial peptides were designed based on the consensus sequence of all known short-chain proline-rich AMPs (Otvos et al., 2005). A homodimer named A3-APO of such a newly designed peptide with 2,4-diaminobutyric acid as a linker was synthesized and examined quite thoroughly for in vitro and in vivo anti-bacterial activity. In the course of these studies, it turned out that the main in vivo activity is associated with a degradation product representing a full single-chain fragment of A3-APO. This fragment named Chex1-Arg20 (Fig. 3.10) was then produced synthetically and tested in mice infected with an extended spectrum β-lactamase (ESBL)-producing *E. coli* strain (Noto et al., 2008). Mice were pretreated with 18 mg/kg cisplatin for 3 days to impair kidney clearance similar to the levels observed in humans before they were challenged by intra-peritoneal injection of the bacteria. For treatment, 10 or 20 mg/kg Chex1-Arg20 were administered intraperitoneally at 4, 8 and 12 h after challenge. Efficacy was assessed by determining blood bacterial counts. Both regimens significantly reduced bacteriemia, with the higher dose being comparably efficient to 40 mg/kg imipenem. A small peptide designed on the basis of the N-terminal part of pyrrhocoricin containing the non-natural amino acid D-cyclohexylalanine (Cha) with the sequence VDKLYChaLPRPT was found to bind to *E. coli* DnaK with a dissociation constant (K_d) of 5.5 nM, the lowest that has ever been reported for a peptide (Liebscher and Roujeinikova, 2009). How this finding will translate into improved AMPs remains to be seen.

3.4.4 Glycine-Rich Polypeptides

The size of the glycine-rich anti-microbial polypeptides varies between 8 kDa (coleoptericin, holotricin, acaloleptins) and 30 kDa (sarcotoxins II). They have been isolated from insects of the orders Diptera (diptericins, attacins, sarcotoxins II), Lepidoptera (attacins, gloverins), Hymenoptera (hymenoptaecins), Coleoptera

Attacins (*Hyalophora cecropia*)

Acidic attacin DAH**G**ALTLNSD**G**TS**G**AVVKVPFA**G**NDKNIVSAI**G**SVDLTDRQKL**G**AATA**G**VALDNIN**G**H**G**LSLTDTHIP**G**
Basic attacin -QA**G**ALTINSD**G**TS**G**AVVKVPIT**G**NENHKFSAL**G**SVDLTNQMKL**G**AATA**G**LAYDN**G**N**G**H**G**ATLTKTHIP**G**

F**G**DKMTAA**G**KVNVFHNDNHDITAKAFATRNMPDIANVPNFNTV**GGG**IDYMFKDKI**G**ASASAAHTDFINRNDYSLD**G**KLNLF
F**G**DKMTAA**G**KVNLFHNDNHDFSAKAFATKNMPNIPQVPNFNTV**G**A**G**VDYMFKDKI**G**ASANAAHTDFINRNDYSL**GG**KLNLF

KTPDTSIDFNA**G**FKKFDTPFMKSSWEPNF**G**FSLSKYF
KTPTTSLDFNA**G**WKKFDTPFFKSSWEPSTSFSFSKYF

Sarcotoxin IIA (*Sarcophaga peregrina*)

QKLPVPIPPPTNPPVAAFHNSVATNSK**GG**QDVSVKLAATNL**G**NKHVQPIAEVFAE**G**NTK**GG**NVLR**G**ATV**G**VQ**G**H**G**L**G**ASVTKS
QDGIAESFRKQAEANLRL**G**DSASLIGKVSQTDTKIK**G**IDFKPQLSSSSLALQ**G**DRL**G**ASISRDVNR**G**VSDTLTKSVSANLFRN
DNHNLDASVFRSDVRQNN**G**FNFQKT**GG**MLDYSHAN**G**H**G**LNA**G**LTRFS**G**I**G**NQATV**GG**YSTLFRSND**G**LTSLKANA**GG**SQWLS**G**
PFANQRDYSF**G**L**G**LSHNAWR*

Hymenoptaecin (*Apis mellifera*)

pEER**G**SIVIQ**G**TKE**G**KSRPSLDIDYKQRVYDKN**G**MT**G**DAY**GG**LNIRP**G**QPSRQHAGFEFGKEYKN**G**FIK**G**QSEVQR**G**P**GG**RLS
PYF**G**IN**GG**FRF

Gloverin (*Hyalophora gloveri*)

DVTWDKNI**G**N**G**KVF**G**TL**G**QNDD**G**LF**G**KA**G**FKQQFFNDDR**G**KFE**G**QAY**G**TRVL**G**PA**GG**TTNF**GG**RLDWSDKNANAALDISKQI**G**
GRPNLSAS**G**A**G**VWDFDKNTRLSA**GG**SLSTM**G**R**G**KPDV**G**VHAQFQHDF

Fig. 3.11 Primary structures of glycine-rich anti-microbial polypeptides of different insects. Glycine residues are in *bold face*. The N-terminal glutamine residue of hymenoptaecin is post-translationally modified to 2-pyrrolidone-5-carboxylic acid (pE)

(coleoptericin, holotricin 2 and 3, tenecin 3, acaloleptins A), and Hemiptera (hemiptericin) (Hetru et al., 1998; Bulet et al., 1999; Imamura et al., 1999).

Attacins, originally identified in the hemolymph of immuno-stimulated diapausing *Hyalophora cecropia* pupae, may be regarded as prototypic glycine-rich anti-microbial polypeptides, with a size of 20 kDa and a more or less equal distribution of the glycine residues over the complete primary structure (Hultmark et al., 1983; Kockum et al., 1984). Two isoforms, an acidic (calculated pI 6.0) and a basic (calculated pI 9.1) attacin with 80% sequence identity were found in *H. cecropia* (Fig. 3.11). Among the three *D. melanogaster* attacins (attacins A–C) attacin C is produced from a pro-protein featuring a peculiar short proline-rich N-terminal domain, that is released upon proteolytic processing (Rabel et al., 2004). The proline-rich peptide, referred to as the maturated pro-domain of attacin C (MPAC), carries an *N*-acetylhexosamine *O*-glycosylation at threonine 16 and thus strikingly resembles the molecular architecture of drosocin and other proline-rich AMPs (Fig. 3.12). Synthetic unglycosylated MPAC and MPAC with a mono- or disaccharide had only very moderate activity against *E. coli* with a tendency to more efficient growth inhibition by the glycosylated forms. The growth of other bacteria, yeast and fungi was unaffected. Nevertheless, MPAC was able to improve the activity of cecropin A. This effect was most evident with *Enterobacter cloacae* as test organism.

The four sarcotoxin II isoforms (sarcotoxins IIA–IID) isolated from the immune hemolymph of third instar larvae of *S. peregrina* are the largest inducible antibacterial insect polypeptides known to date (Ando et al., 1987; Kanai and Natori, 1990). The sarcotoxins may have evolved from an attacin-like ancestor by the addition of a short N-terminal proline-rich domain, which is retained in the mature

*MSKIVLLFVVIVGVLGSLAVALP*QRPYTQPLIYYPPPP***T***PPRIYRARRQVL**GG**SLTSNPS**GG**ADARLDLSKAV**G**TPDHHVI**G**Q
VFAA**G**NTQTKPVSTPVTS**G**ATL**G**YNNH**G**H**G**LELTKTHTP**G**VRDSFQQTATANLFNN**G**VHNLDAKAFASQNQLAN**G**FKFDRN**G**A
ALDYSHIK**G**H**G**ATLTHANIP**G**L**G**KQLEL**GG**RANLWQSQDRNTRLDL**G**STASKWTS**G**PFK**G**QTDL**G**ANL**G**LSHYF**G**

Fig. 3.12 Amino acid sequence of pre-pro-attacin C of *Drosophila melanogaster*. The signal peptide (*italic*) is followed by a pro-domain corresponding to the MPAC peptide (*underlined*) that is acting synergistically with cecopins; the glycosylated threonine residue of MPAC is indicated in *bold italic* type. The mature attacin C starts with the sequence QVLGG. Glycine residues of the mature attacin C are in *bold face*

Protophormia terraenovae	DEKPKLILP***T***PAPPNLTQLV**GGGGG**NRKD**G**F**G**VSVDAHQKVWTSDN**G**RHSI**G**V***T***P**G**YSQH
Drosophila melanogaster	DDMT--MKP***T***PPPQYPLNLQ**GGGGG**QSGD**G**F**G**FAVQ**G**HQKVWTSDN**G**RHEI**G**LN**GG**Y**G**QH
Sarcophaga peregrina	DLH--IPPPDNKINWPQLS**GGGGG**SPKT**G**YDININAQQK
	L**GG**PY**G**NSRPDYRI**G**A**G**YSYNF
	L**GG**PY**G**NSEPSWKV**G**STYTYRFPNF

Fig. 3.13 Primary structures of diptericins of three dipteran species. Glycine residues in *bold face*; *O*-glycosylated threonine residues in *bold italic* type

polypeptide, and an extension of the glycine-rich domain (Asling et al., 1995) (Fig. 3.11).

Also the markedly smaller diptericins consist of a short N-terminal proline-rich domain followed by an extended glycine-rich domain (Fig. 3.13). In contrast to the attacins and sarcotoxins, the glycine-rich domain of the diptericins starts with a peculiar pentaglycine repeat. Experiments with chemically synthesized *Protophormia terraenovae* diptericin and fragments thereof demonstrated that the main anti-bacterial determinant lays in the glycine-rich domain, although the N-terminal proline-rich segment, which alone is inactive, augments the activity by 100-fold (Winans et al., 1999). Diptericin isolated from *P. terraenovae* larvae is *O*-glycosylated at two threonine residues, one in the proline-rich domain (residue 10) and one in the glycine-rich domain (residue 54). The sugar side-chains were identified as two identical trisaccharides [glucose → galactose → *N*-acetylglucosamine → (threonine)] (Bulet et al., 1995). It remains an open possibility that the native diptericin carries more complex glycans as it could not be excluded that sugar bonds were cleaved during the extraction and purification procedure, a concern that also applies to the known glycosylated proline-rich AMPs. Reported results on the essentiality of the glycosylation for anti-bacterial activity are conflicting. In the study by Bulet et al. (1995) the activity of diptericin isolated from *P. terraenovae* larvae was fully lost after enzymatic removal of the carbohydrates. In contrast, the activity of dipericin was consistently independent of its glycosylation state in the study of Winans et al. (1999) carried out with fully synthetic peptides with or without sugar side-chains. As a possible explanation, the synthetic glycoforms carrying only *N*-acetylglucosamine monosaccharide modifications used by Winans et al. (1999) may be less active than the putatively native trisaccharide forms investigated by Bulet et al. (1995). As another possibility, the polypeptide was degraded during enzymatic treatment as a consequence of contaminating proteases (Cudic et al., 1999). Diptericin of *D. melanogaster* is only glycosylated at a threonine residue within the N-terminal proline-rich domain (Imler and Bulet,

Holotrichia diomphalia	Y**G**P**G**D**G**H**GGG**H**GGG**H**GGG**H**G**N**G**Q**GGG**H**G**H**G**P**GGG**F**GGG**H**GGG**H**GGGG**R**GG**--**GG**S**GGGG**S
Tenebrio molitor	----DHHD**G**-HL**GG**HQT**G**HQ**GG**QQ**GG**HL**GG**QQ**GG**HL**GG**HQ**GG**QP**GG**HL**GG**HQ**GG**I**GG**T**GG**
Sarcophaga peregrina	------QH**G**-H**GG**QDQH**G**Y**G**H**G**QQAVY**G**K**G**HE**G**--H**G**VNNL**G**QD**G**H**G**QH**G**----YAH**G**HS
	P**G**H**G**A**GGG**YP**GG**H**GGG**HH**GG**YQTH**G**Y
	QQH**G**QH**G**--P**G**-T**G**A**G**HQ**GG**YKTH**G**H
	DQH**G**H**GG**------QH**G**QHD**G**YKNR**G**Y

Fig. 3.14 Primary structures of the three glycine-rich anti-fungal polypeptides holotricin 3, tenecin 3, and AFP of *H. diomphalia*, *T. molitor*, and *S. peregrina* respectively. Glycine residues in *bold face*

2005), and the *S. peregrina* diptericin apparently is unmodified (Ishikawa et al., 1992). Generally, glycosylation of anti-microbial polypeptides may improve solubility, reduce non-specific protein binding, increase protease resistance, and provide specific clearance mechanisms, properties that mainly would be evident in intact organisms.

In most cases, the anti-microbial activity of the glycine-rich polypeptides is directed against a narrow spectrum of Gram-negative bacteria. However, hymenoptaecin (Fig. 3.11) isolated from the hemolymph of adult honeybees inhibited two Gram-positive bacteria species (*Micrococcus lysodeikticus* and *Bacillus megaterium*) in addition to several Gram-negative bacteria; no activity was seen against *Clostridium difficile*, *Rhodococcus equi*, *Streptococcus B*, and *S. aureus* (Casteels et al., 1993). Activity against yeast is reported for three homologous glycine-rich polypeptides, the anti-fungal protein (AFP) from *S. peregrina*, holotricin 3 from *Holotrichia diomphalia*, and tenecin 3 from *Tenebrio molitor* (Iijima et al., 1993; Lee et al., 1995; Kim et al., 1998) (Fig. 3.14). A more detailed survey of its anti-microbial spectrum revealed that tenecin 3 is active against *Candida albicans* and *Saccharomyces cerevisiae* but neither against Gram-positive nor Gram-negative bacteria (Kim et al., 2001). Furthermore, attacin B from *Hyphantria cunea* was found to be active against *E. coli*, *Citrobacter freundii* and *C. albicans* (Kwon et al., 2008).

Quite little is known about the mode of action of the glycine-rich polypeptides. It was concluded from NMR and CD spectroscopy that *P. terraenovae* diptericin adopts essentially random coil conformations, both in aqueous solution and in the presence of 50% trifluoroethanol as a structure-inducing agent, thus providing no clue to its anti-bacterial mechanism (Cudic et al., 1999). In similar experiments, an unordered secondary structure with very loose turn-like elements not significantly affected by organic solvents or sodium dodecyl sulfate (SDS) micelles was observed for tenecin 3 (Lee et al., 1999). For gloverin (Fig. 11) from *Hyalophora gloveri*, it was shown by CD spectroscopy that this polypeptide undergoes a transition from random coil conformations to a conformation with an approximately 50% α-helix proportion after addition of 20% hexafluoro-isopropanol, but there was no indication for any amphipathic structure (Axén et al., 1997). Treatment of *E. coli* cells with acidic or basic attacin of *H. cecropia* leads to an increase in outer-membrane permeability, preceding any increase in inner-membrane permeability by at least one generation time (approximately 45 min). Only 10 min after exposure, decreased synthesis of the outer-membrane proteins OmpC, OmpF, and OmpA is observed

(Carlsson et al., 1991; Carlsson et al., 1998). Inhibition of the outer-membrane protein synthesis is achieved on the transcriptional level and triggered by binding of attacin to the cell surface without entering the inner membrane or the cytoplasm. Primary binding occurs on LPS, explaining why basic attacin is more active against *E. coli* than its acidic form. Gloverin from *H. gloveri* appears to exert a very similar mechanism as also inhibition of outer-membrane proteins was observed (Axén et al., 1997). The fungicidal tenecin 3 appears to act through a fully unrelated mechanism. Fluorescein isothiocyanate (FITC)-labelled tenecin 3 was internalized into the cytoplasm of susceptible *C. albicans* cells without any evidence for pore formation (Kim et al., 2001). The uptake was temperature dependent and only observed in metabolically active cells. The intra-cellular target remains fully unknown.

With respect to their large size, the narrow spectrum of susceptible organisms and the mostly slow killing kinetics, glycine-rich polypeptides are seemingly less suited as anti-infective lead structures or for application in biotechnology. However, preliminary results indicate that the potential of these polypeptides may have been underestimated. For example, *Proteus mirabilis* and other *Proteus* species are inherently resistant against cationic anti-bacterial substances such as polymyxins or cecropins, most likely due to their specific LPS composition characterized by a high content of phosphate-linked 4-aminoarabinose resulting in a less acidic bacterial surface (Rózalski et al., 1997). Remarkably, a highly polymyxin-resistant *P. mirabilis* strain was found to be very sensitive to the acidic form of *H. cecropia* attacin (Carlsson et al., 1998). The presence of LPS modified with 4-aminoarabinose appears also to be the reason for the well-known polymyxin resistance of *Morganella morganii*, *Providencia rettgeri*, and *Serratia marcescens*. Therefore, to develop attacin-based treatments against these important nosocomial bacteria is an intriguing possibility but remains the subject of future research. Furthermore, since attacins demonstrably kill bacteria without entering the cells, glycine-rich polypeptides may be used for surface modification in order to prevent bacterial colonization. In preliminary experiments by Carlsson et al. (1998) it has already been shown that attacin immobilized to agarose beads retains its bactericidal activity.

3.5 AMPs in Clinical Trials

3.5.1 Human AMPs

Despite a considerable number of pre-clinical experiments, insect AMPs have not yet been tested in humans. Nevertheless, several clinical studies have been performed with artificial peptides obtained by modification and optimization of human, porcine, bovine, and frog AMPs. Human and other mammalian AMPs have been investigated in quite a lot of detail with respect to their immediate medical relevance. In addition, a large part of the currently known AMPs are of amphibian origin as amphibian skin secretions contain high concentrations of AMPs and

various other bioactive molecules such as biogenic amines, complex alkaloids and other pharmacologically active peptides related to mammalian hormones and neurotransmitters (Simmaco et al., 1998). The most prominent mammalian AMPs with broad-spectrum anti-bacterial, anti-fungal, and anti-viral activity are the defensins, characterized by a triple-stranded β-hairpin structure, six conserved disulfide-linked cysteine residues, and a positive net charge. On the basis of the pattern of cysteine pairing two main subfamilies are distinguished, the α- and the β-defensins (Yamasaki and Gallo, 2008). Six α-defensins have been identified in humans, HNP (human neutrophil peptide)-1 to -4, HD-5 and HD-6. HNP-2 is a truncated form of HNP-1 or HNP-3 peptides. HNP-1 to -3 and, in smaller amounts, HNP-4 mainly occur in the azurophilic granules of neutrophils (Lehrer, 2007). HD-5 and HD-6 are mainly secreted from Paneth cells in the small intestine. Four human β-defensins (hBD-1 to -4) have been characterized, that are produced by mucosa and epithelial cells. Cathelicidins are another important family of AMPs in mammals. They are characterized by a conserved N-terminal domain that is proteolytically cleaved to generate the mature, active peptide contained within the C-terminus. The prosequence is highly homologous to cathelin, a cathepsin L inhibitor. The C-terminal domain is diverse with α-helical or disulfide bond-stabilized β-hairpin peptides as well as peptides with unusual amino acid bias all represented (Jenssen et al., 2006). Some mammals, such as cattle, possess multiple cathelicidins. In humans, only one cathelicidin named LL-37 exists. LL-37 represents an α-helix-type AMP that is mainly found in the exocytosed material of neutrophils and as smaller fragments on the skin surface (Yamasaki and Gallo, 2008). Dermcidin is a human AMP with broad-spectrum anti-microbial activity constitutively secreted by eccrine sweat glands (Schittek et al., 2001). In healthy human skin, another AMP, psoriasin/S100A7, is focally produced in areas with high bacterial colonization such as the uppermost parts of the hair follicles, the nose, and the palms of the hands (Schröder and Harder, 2006). The pronounced activity of psoriasin against *E. coli* is probably the most important reason why human skin is rarely infected by this gut bacterium. Various further human peptides and proteins with anti-microbial properties exist that, very often, were first known for other functions. For example, RNase 7 is currently considered the perhaps most important component of the chemical skin barrier (Boix and Nogués, 2007). The small, positively charged protein (14.5 kDa) is able to permeate the bacterial membrane and kills Gram-positive and Gram-negative bacteria and yeast. Its ribonucleolytic activity is dispensable for anti-microbial activity. In addition to their anti-microbial activity, mammalian AMPs typically also display immunomodulatory, hormone and/or growth factor-like activity. For example, human α- and β-defensins are known to modify cell migration and maturation, induce cytokines and trigger histamin and prostaglandin D2 release from mast cells (Yamasaki and Gallo, 2008). Further, α-defensins and LL-37 stimulate cell growth in a narrow concentration range, while being cytotoxic at higher concentration (Aarbiou et al., 2006). Their pleiotropic effects may have been the reason why the best known human AMPs, the defensins and LL-37, have not been considered for clinical trials. Instead, quite remote human AMPs or anti-bacterial proteins have served as templates for clinical trial candidates. As detailed in the

Table 3.1 Overview of AMPs tested in clinical studies

Clinical trial candidate	Parent AMP	Indication	Outcome
UBI 29-41	Ubiquicidin	Radioactive probe for whole-body imaging	Successful identification of sites of inflammation
$rBPI_{21}$	BPI[a]	Fulminant meningococcal sepsis	Reduction in mortality (not significant, probably due to inappropriate study design)
P-113	Histatins	Experimental gingivitis	Improvement in certain dose groups and certain populations of volunteers
hLF1-11	Lactoferrin	Bacterial hospital infections	Safety in humans established
Pexiganan	Magainins	Diabetic foot ulcers	Comparably effective to standard ofloxacin therapy
Iseganan	Protegrins	Stomatitis associated with anti-cancer chemotherapy	No efficacy
		Oral mucositis associated with radiotherapy	No efficacy
		Ventilator-associated pneumonia	No efficacy
Omiganan	Indolicidin	Acne	Significant improvement compared to placebo
		Central catheter-related bloodstream infections	Significantly superior to povidone iodine in reduction of catheter colonization and catheter-related local site infections

[a]Bactericidal/permeability increasing protein

following paragraphs, these include ubiquicidin, bactericidal/permeability increasing protein (BPI), histatins, and lactoferrin (Table 3.1). In addition, clinical studies have been performed with peptides derived from the α-helical magainins from frog skin, the porcine cathelicidins known as protegrins, and the tryptophan-rich bovine cathelicidin named indolicidin.

3.5.2 UBI 29-41 Derived from Human Ubiquicidin

An anti-microbial peptide originally isolated from murine macrophages was termed ubiquicidin (Hiemstra et al., 1999). It was found that ubiquicidin is identical to the ribosomal protein S30, which is incorporated into the small ribosomal subunit. Mouse and human ubiquicidin/S30 share full amino acid sequence identity. Ubiquicidin/S30 is a small cationic protein consisting of 59 amino acid residues. It is produced by post-translational processing of the Fau protein, which consists of an N-terminal domain with homology to ubiquitin (36% identity) and the C-terminal

ubiquicidin/S30 domain (Kas et al., 1992). The ubiquitin-like domain may function as a substituent or inhibitor of ubiquitin and was found to be a subunit of the murine monoclonal non-specific suppressor factor (MNSF) (Herrmann et al., 2007). While AMPs of phagocytes are typically stored in granules and transferred to the phagolysosome or secreted in the extra-cellular environment, ubiquicidin is present in the cytosolic fraction and may play a role in inhibiting bacteria able to grow in the host cell cytosol such as *L. monocytogenes*, *Shigella* spp., and *Rickettsia* spp. Little is known about the physiological relevance, mechanism of activity, and anti-microbial spectrum of ubiquicidin. Nevertheless, full-length ubiquicidin (UBI 1-59) injected intravenously was effective in a methicillin resistant *S. aureus* (MRSA) mouse model, and only somewhat reduced activity was observed with a derived synthetic peptide consisting of only eight amino acid residues (UBI 31–38) (Brouwer et al., 2006). Moreover, another ubiquicidin-derived peptide (UBI 29–41) was labelled with radioactive technetium-99 m (^{99m}Tc) and used as a probe for real-time, whole-body imaging for the scintigraphic visualization of infected tissues in mice (Brouwer et al., 2008). In an early clinical study including seven adult patients with suspected bone or soft tissue infection this procedure enabled identification of the sites of inflammation (Gandomkar et al., 2009).

3.5.3 rBPI$_{21}$ Derived from Human Bactericidal/Permeability Increasing Protein (BPI)

Bactericidal/permeability increasing protein (BPI) is a major constituent of human neutrophils (0.5–1% of total protein) and, to a lesser extent, also present in eosinophils (Elsbach, 1998; Schultz and Weiss, 2007). In addition, epithelial cells of the oral, gastrointestinal and female genital tracts are able to produce BPI (Canny et al., 2006). The cationic 55 kDa protein displays activity against a wide range of Gram-negative bacteria at nanomolar concentration, reflecting high affinity to the lipid A moiety of lipopolysaccharides (LPS) and potent endotoxin-neutralizing activity. Patients with Crohn's disease and cystic fibrosis often have anti-neutrophil cytoplasmic antibodies (ANCAs) against BPI, potentially resulting in BPI activity deficiency (Schinke et al., 2004). The endotoxin-neutralizing activity of a 21-kDa recombinant protein (rBPI$_{21}$) derived from the N-terminal domain of BPI has been evaluated in clinical studies. In an open-label, dose-escalation phase I/II trial, 26 pediatric patients with fulminant meningococcal sepsis were treated with rBPI$_{21}$ administered intravenously. Only one of the patients (4%) died, which favorably compared to a historical control group with 20% mortality (11 of 54 patients) (Giroir et al., 1997). In a subsequent phase III, randomized, placebo-controlled trial, 190 patients received rBPI$_{21}$ and 203 placebo (Levin et al., 2000). As an outcome, the beneficial effect of rBPI$_{21}$ could be confirmed, however reduction in mortality did not reach statistical significance, probably because the study was underpowered by an inappropriate design not taking into account the high mortality of patients in the time between recruitment and drug administration (Giroir et al., 2001).

3.5.4 P-113 Derived from Human Histatins

A number of histidine-rich cationic peptides ranging in size from 7 to 38 amino acid residues collectively called histatins are present in human saliva (Tsai and Bobek, 1998). They are secreted by the parotid and sub-mandibular/sub-lingual salivary glands. The most prominent human histatins are histatin 1, 3, and 5 consisting of 38, 32, and 24 residues, respectively (Campese et al., 2009). Histatins 1 and 3 are products of different genes while histatin 5 is a proteolytic cleavage product of histatin 3 (Oppenheim et al., 1988). Histatins display broad-spectrum anti-microbial activity and are particularly active against the yeast *C. albicans*, with histatin 5 being the most potent. In AIDS patients, increased *C. albicans* colonization was correlated with decreased histatin 5 concentrations possibly as the result of salivary gland dysfunction (Torres et al., 2009). Quite recently, histatins were identified as the major wound-closure stimulating factors in human saliva (Oudhoff et al., 2008). While the prominent anti-microbial histatin 5 was inactive, wound-closure activity was observed with histatins 1, 2, and 3. Most active was histatin 2, a histatin 1 variant without the N-terminal part. Furthermore, histatin 1 has been implicated in the maintenance of tooth enamel mineral and pellicle formation (Sun et al., 2009).

A 12-amino acid fragment of histatin 5, designated P-113, has been identified as the smallest fragment that retains anti-microbial activity. P-113 was evaluated in a single-center, randomized, double-blind, parallel-design clinical phase I/II study for the treatment of experimental gingivitis (Mickels et al., 2001). Prior to drug administration, volunteers were brought to gingival health by scaling, tooth brushing, and oral hygiene instructions. A total of 159 subjects were randomly assigned to one of the following treatment arms: 0.005% P-113 mouthrinse, 0.01% P-113 mouthrinse, 0.05% P-113 mouthrinse, or placebo mouthrinse. Subjects self-administered 15 ml of study mouthrinse twice daily for 28 days. During this time subjects were instructed to abstain from all oral hygiene procedures. Efficacy was assessed by evaluation for plaque index, gingival index, and bleeding on probing. As result, a significant reduction in plaque and a reduction in gingivitis were observed for patients using 0.01% P-113 mouthrinse. There was also a trend towards reduction of bleeding on probing in the 0.01% dose group. No treatment-related adverse events and no adverse shift in supra-gingival microflora were reported.

In a subsequent phase IIb multi-center clinical study of similar design, P-113 was evaluated as 0.01 and 0.03% mouthrinse versus placebo in 294 healthy volunteers (Van Dyke et al., 2002). In this study a significant reduction of bleeding on probing was found in the 0.01% group, and there were non-significant trends in reduction of the other parameters. A subpopulation of subjects was identified who suffered from gingival inflammation just prior to administration of mouthrinse, despite rigorous oral hygiene procedures in the pre-treatment phase. In these subjects particularly prone to the development of gingivitis significant findings were observed for all parameters, in particular if smokers were excluded from the evaluation.

In a further clinical phase II study using the human gingivitis model, a topical P-113 gel formulation was tested at three different concentrations (0.0625, 0.125, and 0.375%) in 106 subjects (Paquette et al., 2002). Significant reduction

of bleeding on probing was observed in all dose groups. Inter-group differences for plaque index or gingival index were borderline or nonsignificant, respectively.

In addition to the clinical studies, the possibility was investigated to manufacture histatin 5 impregnated denture resins in order to prevent denture-induced stomatitis which is associated with high levels of *C. albicans* adhesion (Edgerton et al., 1995). Poly(methyl methacrylate) (PMMA), the common material used for manufacturing of dentures, was modified by surface polymerization of methyl methacrylic acid, which allowed adsorption of histatin 5. In an in vitro adhesion assay, the modified resin reduced human saliva-mediated adherence of yeast cells to the polymer.

3.5.5 hLF1-11 Derived from Human Lactoferrin

Lactoferrin is a mammalian iron-binding glycoprotein of ca. 80 kDa belonging to the transferrin family (Levay and Viljoen 1995). In contrast to transferrin, which is primarily present in the bloodstream where it functions in iron delivery to cells, lactoferrin is mostly found in exocrine secretion, and notably in secondary granules of neutrophils. Rich sources of lactoferrin are milk (1–2 mg/ml), colostral milk (3–7 mg/ml), tear fluid (2 mg/ml), and seminal plasma (1 mg/ml). In breast-fed infants lactoferrin may improve iron availability (Lönnerdal, 2009). As its primary function, however, lactoferrin is commonly considered to play a role in maternal and innate immunity (Jenssen and Hancock, 2009). It has been shown that lactoferrin is active against bacteria, viruses, fungi, and parasites, and it has been implicated in protection against cancer. It is well established that lactoferrin is a multi-functional protein with the main activities residing in particular domains. Sequestration of iron, thus depriving microorganisms of the iron essential for growth, is one important mechanism. In addition, the positively charged helix at the N-terminus described as the lactoferricin domain is important for the bactericidal activity. Proteolysis in the stomach leads to the release of the lactoferricin peptide (25 residues), which is more active against most susceptible microorganisms than the native lactoferrin and probably acts by the formation of amphiphatic structures leading to membrane disruption (van der Kraan et al., 2004). The N-terminal part of lactoferrin also possesses a serine protease-like activity which functions to inactivate the type III secretion system used by bacteria to secrete virulence proteins for host cell invasion. The antiviral activity of lactoferrin depends on blocking of host cell glycosaminoglycans, mainly heparan sulfate, used by the virus for adsorption, or on direct interaction between lactoferrin and the viral particle. Possibly, the glycan chains of lactoferrin are essential for the latter mechanism. The anti-carcinogenic effect of lactoferrin and lactoferrin-derived peptides appears to be mediated by various immunomodulatory properties and its ability to promote apoptosis in cancer cells (Iigo et al., 2009).

A peptide representing the first 11 amino acid residues of human lactoferrin (hLF1-11) was demonstrated in vitro to be exceptionally active against a variety of bacteria and fungi including fluconazole-resistant *C. albicans* (Lupetti et al., 2003). In a murine thigh muscle infection model, hLF1-11 was effective against both multi-drug-resistant *Acinetobacter baumanii* and methicillin-resistant *S. aureus*

(MRSA) (Dijkshoorn et al., 2004; Brouwer and Welling, 2008). Furthermore, hLF1-11 released from biodegradable calcium phosphate cement was successfully applied to prevent experimental osteomyelitis caused by *S. aureus* in rabbits (Stallmann et al., 2004). A clinical phase I study on hLF1-11 as potential treatment for bacterial hospital infections reportedly has been completed. It is stated that "overall safety has been established in pre-clinical and clinical studies" (http://www.am-pharma.com).

3.5.6 Pexiganan Derived from Frog Magainins

Magainins are cationic α-helical peptides, 21–27 residues in length, isolated from the skin of the African clawed frog *Xenopus laevis* with broad-spectrum activity towards Gram-positive and Gram-negative bacteria, fungi, protozoa, viruses, and tumor cells (Zasloff, 1987). A synthetic 22-residue analogue of magainin 2 was named pexiganan (or MSI-78). Pexiganan was evaluated for the treatment of mildly infected diabetic foot ulcers in two consecutive, double-blind, controlled trials (Lamb and Wiseman, 1998; Lipsky et al., 2008). Overall, 835 outpatients were randomized to topical treatment with pexiganan (1% pexiganan acetate cream twice daily) or systemic treatment with an oral fluoroquinolone antibiotic (200 mg ofloxacin twice daily), plus a respective inactive placebo. Although the first study failed to demonstrate equivalence, the second study and the combined date for both trials indicated equivalent efficacy of topical pexiganan and oral ofloxacin with respect to clinical improvement rates, microbiological eradication rates, and wound healing rates. Bacterial resistance emerged in some patients who received ofloxacin, but no significant resistance development was observed in the pexiganan group. It was concluded that topical treatment with the peptide reduces the risk of selecting anti-microbial-resistant bacteria and avoids the adverse effects of systemic anti-infective agents. However, the FDA rejected the approval of pexiganan in 1999, reportedly as it did not offer any great advantage over the current standard of care (Marr et al., 2006).

3.5.7 Iseganan Derived from Porcine Protegrins

The protegrins, first isolated from porcine neutrophils, are members of the cathelicidin family (Kokryakov et al., 1993). Five natural protegrins, 16–18 residues long are known and were shown to be active against Gram-negatives, Gram-positives, and yeast. The highly cationic peptides form two intra-molecular disulfide bonds and adopt an amphiphilic structure composed of an anti-parallel β-sheet with a β-turn. Iseganan (also named IB-367) was identified as a synthetic protegrin analogue with broad-spectrum anti-microbial activity (Chen et al., 2000). A phase III, randomized, double-blind, placebo-controlled study was conducted to evaluate the efficacy of iseganan in the reduction of stomatitis in patients receiving stomatotoxic anti-cancer chemotherapy (Giles et al., 2004). A total of 502 patients were treated with iseganan, 9 mg per dose, or placebo, administered as an oral rinse six times daily.

As an outcome of the study, a major impact of iseganan on reducing stomatitis was not detected. In a similar study, iseganan was assessed in a total of 545 patients for reducing the severity of oral mucositis during the course of radiotherapy treatment of head-and-neck cancer (Trotti et al., 2004). However, no beneficial effect of iseganan compared to placebo was found. Also, no beneficial effect of iseganan in preventing ventilator-associated pneumonia was observed in a placebo-controlled study including 709 intensive care patients (Kollef et al., 2006). It was suggested that the lack of efficacy of iseganan may have been caused by an inappropriate pharmaceutical formulation (van Saene et al., 2007). For future studies the use of a mucosa-adhesive paste instead of the aqueous solution was proposed.

3.5.8 Omiganan Derived from Bovine Indolicidin

The indolicidin derivative omiganan is currently considered to represent the AMP in the most advanced stage of clinical development. Indolicidin is a member of the cathelicidin family in cattle first isolated from bovine neutrophils (Selsted et al., 1992). The 13-residue AMP contains five tryptophan residues (39%), thus displaying the highest tryptophan proportion ever seen in a peptide or protein (Chan et al., 2006). Upon interaction with biological membranes indolicidin assumes a wedge-type or boat-shaped conformation with the hydrophobic tryptophan residues in the wedge's trough, flanked by positively charged regions at both ends. If fully extended, indolicidin is able to span both membrane leaflets, despite only consisting of 13 amino acid residues. This is possible because indolicidin adopts an approximate poly-L-proline type II helix conformation characterized by very high helical pitches. The exact mode of action of indolicidin is still unknown. At bactericidal concentrations the peptide appears to translocate through the membrane into the cytosol where it may bind to nucleic acids, while it is membranolytic at higher concentrations. Indolicidin has a very wide killing spectrum with demonstrated activity against Gram-positive and Gram-negative bacteria, fungi, protozoa, and HIV-1. However, its clinical usefulness is compromised by pronounced hemolytic activity, indicative for non-specific disruption of mammalian membranes. This disadvantage appears to be largely eliminated in omiganan, which reportedly is much less hemolytic (Melo et al., 2006). Clinical studies on omiganan have been performed for the treatment of acne and for preventing catheter-related infections (Melo et al., 2006; http://www.migenix.com).

Omiganan administered as a topical solution (MBI 594AN) has been evaluated in five clinical trials for the treatment of acne. Three phase I studies were performed in a total of 36 healthy volunteers and 35 acne patients demonstrating that omiganan is well tolerated and not systemically absorbed after topical administration. A 6-week randomized, double-blind phase IIa study was performed in 75 subjects with facial acne vulgaris. Omiganan diminished the severity of acne in subjects with mild to moderate disease, as shown by reduced counts of inflammatory lesions (papules and pustules; 39% compared to 21% reduction in the omiganan and placebo group, respectively) and non-inflammatory lesions (comedones; 25%

compared to 10% reduction in the omiganan and placebo group, respectively). According to Physician's Global Severity Assessment scores, good to excellent improvement was seen in 41% of patients treated with omiganan compared to 32% for placebo-treated subjects. Treatment with a 2.5% omiganan solution performed equal or superior to a 5% treatment group.

A phase IIb randomized, double-blind, vehicle-controlled dose-finding study was conducted in 241 subjects to evaluate the efficacy and safety of omiganan solutions over a treatment period of 12 weeks. After 6 weeks of treatment, a 2.5% topical solution proved to be statistically superior to the vehicle control in reducing all types of acne lesions measured, specifically reducing inflammatory lesions by 40%. Between 6 and 12 weeks, the control group receiving the vehicle alone displayed a gradual decrease in lesion counts, making the analysis beyond 6 weeks not statistically significant. This was interpreted as a placebo effect seen frequently in acne studies. In the group receiving 1.25% omiganan solution, a dose response was seen showing trends towards efficacy.

A phase III randomized, evaluation-committee-blinded study was performed in 1,400 patients to demonstrate that omiganan is superior to povidone iodine in preventing central catheter-related bloodstream infections. Omiganan was formulated as 1% gel (MX-226) and administered at the catheter insertion site. The treatment with omiganan achieved a clear, statistically significant reduction in the two secondary efficacy endpoints, catheter colonization (21% reduction) and catheter-related local site infections (49% reduction), compared to povidone iodine. The primary endpoint, a reduction in catheter-related bloodstream infections, resulted in a 15% improvement as compared to povidone iodine, but this effect was not statistically significant due to the small number of patients who contracted infections in this trial (Jenssen et al., 2006).

3.6 Insect AMPs as New Leads for Human Treatments

Although drugs derived from ribosomally synthesized AMPs have not yet been approved, their therapeutic potential has been substantiated in previous clinical studies. Compared with conventional antibiotics, the most potent AMPs investigated so far display unusually broad spectra of activity against Gram-negative and Gram-positive bacteria, fungi, and some viruses. The action of most AMPs is extremely rapid with bactericidal effects observed at concentrations very close to minimal inhibitory concentrations. The ability of AMPs to kill multi-drug-resistant pathogens is one of their major strengths and, as a consequence of their relatively non-specific mechanism, resistance cannot be directly selected. For resistance development, bacterial membranes would have to undergo profound structural and functional changes, as the overall anionic charge of the membrane primarily targeted by AMPs is a fundamental characteristic of bacterial cells.

Nevertheless, in the process of co-evolution pathogens have acquired the ability to sense and to respond to AMPs (Yount and Yeaman, 2005). Altered gene

expression is observed in the Gram-negative bacterium *Salmonella enterica* serovar Typhimurium after exposure to sub-inhibitory concentration of several cationic AMPs leading to modification of outer membrane lipopolysaccharide. Among Gram-positive bacteria, upregulation of proteins involved in cell surface charge has been observed in *Staphylococcus epidermidis* and *S. aureus* in response to multiple AMPs. Proteolytic inactivation of AMPs appears to be a generalized mechanism employed by various pathogens. Staphylococci have been shown to exhibit proteolytic activity against dermcidin after exposure to this AMP present on human skin. *C. albicans* produces a family of ten proteolytic enzymes known as the secreted aspartic proteases (Saps), which have been implicated in adherence, tissue damage, and immune evasion including degradation of defense proteins such as lactoferrin and immunoglobulins. The most recently discovered members, Sap 9 and Sap 10 are bound to the fungal cell membrane and cell wall by glycosylphosphatidylinositol (GPI)-anchors. Histatin 5, an AMP from human saliva, was identified as the first specific host substrate of Sap 9 and Sap 10, with Sap 10 being significantly less active (Meiller et al., 2009). It was suggested that the success of the anti-HIV HAART (highly active anti-retroviral therapy) regimen in reducing the incidence of oral candidiasis may not only result from inhibition of HIV proteases but also from inhibition of Sap 9, thus protecting histatins from degradation. In group A streptococci (*Streptococcus pyogenes* or GAS) the human cathelicidin LL-37 stimulates the production of hyaluronic acid capsules, the notorious virulence determinant of this pathogen, in addition to two other virulence factors, the IL-8 protease Prts/ScpC and the integrin-like/IgG protease Mac/IdeS (Gryllos et al., 2008). Thus, the production of LL-37 in response to GAS infection, paradoxically, increases GAS pathogenicity.

As they are evolutionarily distant from their mammalian counterparts, insect AMPs are less likely to be counteracted by resistance mechanisms existing in pathogens adapted to the human host. In addition, insect AMPs are not expected to display any specific physiological effects in humans besides their anti-microbial activity. Given that only a comparably small number of insect species have been

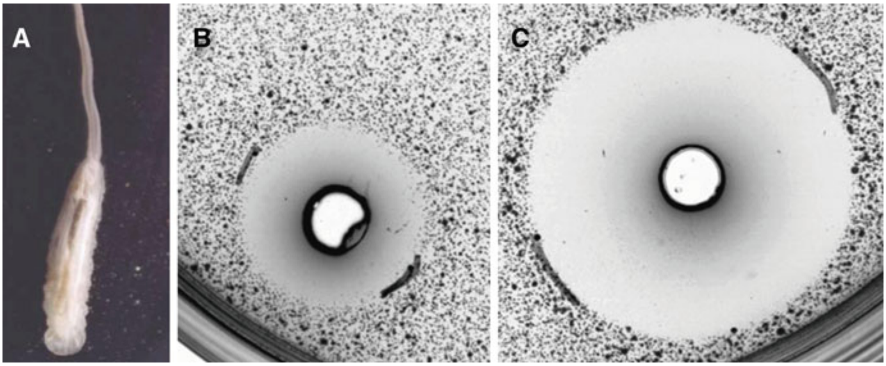


Fig. 3.15 The rat-tailed maggot of the drone fly *Eristalis tenax* as a source of new AMPs. **a** Rat-tailed maggot exhibiting its characteristic snorkel used for air breathing from the pond surface. **b** Inhibition zone assay performed with the hemolymph of non-stimulated maggots. **c** Inhibition zone assay after immune challenge

investigated so far it can be expected that combining state-of-the-art molecular technologies with a knowledge-based entomological approach will result in an exponentially growing number of new AMPs. In recent studies suppressive subtractive hybridization (SSH) proved to be a powerful tool for the discovery of AMPs (Altincicek and Vilcinskas, 2009). Using this technique, 19 new AMPs were identified in rat-tailed maggots of the drone fly *Eristalis tenax* which are capable of surviving in extremely polluted aquatic habitats such as in farmyard liquid manure storage pits (Altincicek and Vilcinskas, 2007) (Fig. 3.15). Remarkably, the hemolymph of non-stimulated maggots already displays considerable antibacterial activity which is further augmented after immune challenge. As the SSH method retrieves mainly non-constitutively expressed genes that are massively upregulated after induction, the assembly of AMPs of *E. tenax* may be even richer. Therefore, current work, applying the new pyrosequencing methodology, aims at whole-transcriptome analysis of *E. tenax*, in addition to other insects known to be highly resistant to microbial stress.

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Chapter 4
From Traditional Maggot Therapy to Modern Biosurgery

Andreas Vilcinskas

Abstract The definition of insect biotechnology provided in this book refers to any technological application that uses insects or derivatives thereof, to make or modify products or processes for specific use in agriculture, food science, and medicine. The most prominent application of insects or insect-derived molecules in medicine is known as maggot therapy or biosurgery. The term maggot therapy has been established for the application of live larvae from the green blow fly *Lucilia sericata* (Diptera: Calliphoridae) to cure open, infected, chronic and/or necrotic wounds, particularly those associated with diabetic or vascular ulcers, whose cure by conventional approaches often fails. This simple and highly successful therapy has received renewed attention in wound therapy because it combines a number of advantages such as efficacy, excellent safety record, and low costs. However, the maggots are sometimes negatively perceived by patients and their sharp mouth-hooks and spicules can sometimes cause pain. Recent research focuses on the identification of molecules from *L. sericata* mediating promotion of wound healing as well as debridement and disinfection of wounds. The production and application of corresponding synthetic or recombinant analogues may expand the use of insect-derived molecules beyond maggot therapy of wounds to include the cure of other diseases by biosurgery.

Keywords Maggot therapy · Biosurgery · Wound healing · Antimicrobials · *Lucilia sericata*

4.1 Renewed Attention to an Old-Fashioned Therapy

Our immune system provides an array of sophisticated mechanisms to combat microorganisms colonizing wounds, but patients with weakened immunity or with

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diseases associated with a prevalence for chronic wounds such as diabetes exhibit a slower healing process and, in the worst case, also non-healing wounds. Chronic and/or non-healing wounds are susceptible to infection with pathogenic bacteria, among which the spread of antibiotic resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* represent an increasing threat for human health. In particular, these hospital-acquired pathogens are difficult to control. Patients infected with multi-drug resistant bacteria suffer additionally from aggressive treatment and their necessary isolation to prevent both super-infections and the spread of germs. Slow and non-healing wounds, particularly when infected, require good nursery care and expensive treatment. Past decades have witnessed an increasing world-wide spread of hospital-acquired pathogens exhibiting resistance against one or more clinically applied antibiotics. This serious dilemma attracted renewed attention to an old-fashioned and almost forgotten therapy.

The use of fly maggots for wound cleansing belongs to the repertoire of traditional medicine in many human cultures around the world such as the Australian Aborigines, Burmese and Chinese hill people, and Central American Mayan Indians (Church, 1996). Observations made on the battle fields of the Napoleonic Wars attracted the attention of modern medicine in maggot-mediated wound-care. The first documented clinical application of maggots was carried out by J.F. Zacharias who tried to help wounded soldiers during the American Civil War. Similarly, the American orthopedic surgeon William Baer used maggots during the First World War. He and his student Stanton Livingstone conducted after the war the first clinical trials and are therefore considered as the pioneers of biosurgery (Mumcuoglu et al., 1999; Thomas et al., 1996). However, interest in their work was lost when modern antibiotics emerged and became available.

4.2 Biology of Medicinal Maggots

The larvae of flies, called maggots, lack a well-defined head and legs. Maggots of species used for medical purposes belong to the blowflies (Diptera: Calliphoridae) whose ancestral life style is believed to be saprophagous using carrion and vertebrate carcasses as habitats for reproduction. Species diversification within Calliphoridae was accompanied by numerous habitat and life style shifts. Several independent switches from saprophytic to other life styles have occurred. For example, herbivorous species seem to originate from those feeding on rotten plants. Radiation within the blowflies resulted also in evolution of insect or mollusk parasitoids. Parasitic maggots infesting humans or other vertebrates originate probably from species specialized on vertebrate carrion or carcasses. Several species have evolved the ability to feed on tissue or liquid body-substances, or ingested food of the host causing infestation defined as myiasis (Zumpt, 1965). Obligate dipteran parasites such as the screwworms reproduce only on living hosts, while facultative ones such as medicinal maggots can develop either on carrion or on live hosts.

Maggot therapy can be defined as artificially induced myiasis, which is carefully controlled to yield positive effects in removal of necrotic tissue and to avoid negative effects on healthy tissues, for example if too many maggots are applied (Sherman et al., 2000). The relationship between fly larvae causing myiasis and their hosts is artificially shifted in maggot therapy from parasitic to beneficial mutualism within the symbiotic continuum. Humans with chronic or non-healing wounds benefit from the therapeutic effects of the maggots, which in turn profit from the availability of necrotic tissue as a favored source of nutrients. Only the larvae of a few facultative parasitic fly species can be used for maggot therapy among the huge number of species causing vertebrate myiasis. The properties of the favorite candidate species have been summarized by Sherman et al. (2000). The "greenbottle" blowfly *Lucilia sericata* has become the predominant species used in clinical maggot therapy today because it fulfills the criteria established for the evaluation of suited candidate species: its maggots are easy to rear in vitro, congregate within cutaneous tissues, feed on necrotic tissue, do not invade internal organs and develop rapidly within the host. Unlike other myiasis-causing members reproducing by laying first instar larvae, which are difficult to handle, *L. sericata* females lay eggs, which can be easily sterilized prior to application. Furthermore, *L. sericata* exhibits limited host specificity, which is beneficial regarding its suitability for maggot therapy. Interestingly, maggots of this fly are serious sheep pests causing "sheep strike" which can be lethal in cases of heavy infestations (Sherman et al., 2000).

4.3 Beneficial Effects of Maggot Therapy

4.3.1 Debridement

The removal of necrotic tissue, called debridement, is an essential mechanism in wound healing. Medical cleaning of wounds causes often larger wounds because surgeons are not as precise as maggots in cutting out necrotic tissue. *L. sericata* maggots applied to wounds are usually smaller than a grain of rice and grow up to more than one centimeter in length by the time they are removed. Their sharp mouth-hooks represent microscopically small tools that accurately dissect necrotic tissues from healthy ones, making medicinal maggots true biosurgeons. Biosurgery can be expanded beyond mechanical removal of rotten tissue to include its digestion by secreted enzymes. Particularly, extra-cellular matrix components that are normally resistant to proteolytic degradation require specialized enzymes for their hydrolysis. A prominent feature of *L. sericata* maggots is that they are able to secrete enzymes capable of digesting necrotic tissues without harming healthy ones (Chambers et al., 2003). Strikingly, these enzymes seem not to be active upon contact with healthy tissues. Candidate genes have recently been identified encoding enzymes providing this sophisticated therapeutic effect (Altincicek and Vilcinskas, 2009), and trials to use maggot-derived proteolytic enzymes formulated in hydrogels are presently under way.

4.4 Promotion of Wound Healing

Mechanical stimulation by the mouth-hook and spiculae of medicinal maggots seems to promote formation of granulation tissue (Beasley and Hirst, 2004). In addition, maggot-derived proteolytic enzymes, which can digest extra-cellular matrix components such as collagen and fibronectin, have been implicated to contribute beyond debridement also to acceleration of wound healing, because their activity may generate bioactive fragments. Furthermore, they were shown to alter the behavior of human dermal fibroblasts, which play a key role in tissue regeneration. Treatment of fibroblasts with *L. sericata* secretions modifies fibroblast adhesion and cell spreading by digestion of fibronectin in the extra-cellular matrix, while keeping cells viable (Horobin et al., 2003). Maggot secretions inhibit pro-inflammatory response of human neutrophils (van der Plas et al., 2007) and monocytes through elevation of cyclic AMP (van der Plas et al., 2009a). Furthermore, it has recently been shown that the secretions skew monocyte-macrophage differentiation away from a pro-inflammatory to a pro-angiogenic type. The increased pro-angiogenic activity of anti-inflammatory macrophages may promote neovascularization and concurrent formation of granular tissue (van der Plas et al., 2009b). Other components secreted by the maggots such as allantoin have also been suggested to play a beneficial role in wound healing.

Clinical studies have demonstrated that maggots can accelerate the healing process thereby reducing duration of therapy from approximately 3 months to 5 days. This up to 18-times faster treatment can reduce the costs per patient from 2,200 to 300 pounds sterling.

4.5 Disinfection

Maggot therapy provides three distinguishable kinds of wound disinfection. First, mechanical stimulation of the tissue surrounding a wound by moving maggots triggers the formation of serous exudate, which contributes to wound cleansing and dilution of bacteria. Second, microbial load of wounds is significantly reduced by maggots, since bacteria are part of their diet, besides necrotic tissue, and are killed via passage through the gut. Third, plausibly to aid killing of bacteria before their ingestion, medicinal maggots secrete potent anti-microbial components into the wound, which are also capable of disinfecting wounds from bacteria that do not respond to commercially available antibiotics such as methicillin-resistant *S. aureus* (MRSA) (Bexfield et al., 2004). It is noteworthy in this regard that maggot therapy does not exclude concurrent application of antibiotics, because they do not affect maggot development within the wound (Sherman et al., 1995).

Harmful bacterial colonizing wounds such as *S. aureus* and *P. aeruginosa* can protect themselves from both the host immune system and therapeutic antibiotics by formation of so-called biofilms. Bacteria residing in biofilms exhibit adapted growth patterns and expression profiles when compared with free-living bacteria. Bacterial components released from biofilms will continuously attract host immune

cells such as neutrophils to the wound which secrete, upon activation, compounds contributing to anti-microbial defense like reactive oxygen species and proteases. The latter can, in turn, cause collateral damage to the tissue surrounding the wound, and thereby inhibit wound healing. Consequently, degradation of bacterial biofilms has emerged as a complementary strategy in therapy of infections. Strikingly, Nibbering and coworkers have recently determined factors in secretions of *L. sericata*, which can break down biofilms produced by *S. aureus* and to a lesser extent also those produced by *P. aeruginosa*. Obviously, modulation of biofilms involves neither bacterial killing nor does it affect their quorum sensing systems (van der Plas et al., 2008).

4.6 Application of Medicinal Maggots

Early techniques for producing and applying medicinal maggots have been reviewed by Sherman et al., 2000. Today, farming of *L. sericata* maggots from sterilized eggs and providing them in dressings or bags ready for application has become a profitable business since maggot therapy has been approved and prospers in many countries. ZooBiotic Ltd., considered as a pioneer of maggot therapy, supplies up to 3,800 hospitals in the UK with sterile maggots marketed as LarvE. In Germany, Biomonde is the leading company producing Biobags containing maggots, which can be directly applied to wounds (Fig. 4.1). The mass production of medicinal maggots under sterile conditions prevents contamination of wounds by transmitted microbes. The dressings and bags are designed to keep the maggots within the wound while allowing both access of air (maggots have to breathe) and drainage of liquefied necrotic tissue and serous exudate. Neither significant risks nor adverse effects resulting from clinical application of medicinal maggots have been reported in the literature (Sherman et al., 2000).

Medicinal maggots are used to cure a variety of necrotic wound types such as infected surgical wounds, pressure sores, and leg ulcers. The number of maggots required for successful therapy depends on their age and size as well as on the size of the wound and its content of necrotic tissue. As a rule, five to ten maggots per cm^2 yield beneficial effects without negative side-effects. The treatment can easily be controlled and terminated just by removal of the outer dressing and collection of escaping maggots. Dressings, bags, and collected maggots have to be disposed of in a safe manner like other potential infectious waste in a hospital.

4.7 Maggot-Derived Compounds with Therapeutic Potential in Biosurgery

4.7.1 Anti-Microbial Molecules from L. sericata

The presence of heat-stable, protease-resistant factors in secretions of *L. sericata* exhibiting activity against MRSA was first reported by Bexfield et al. (2004).

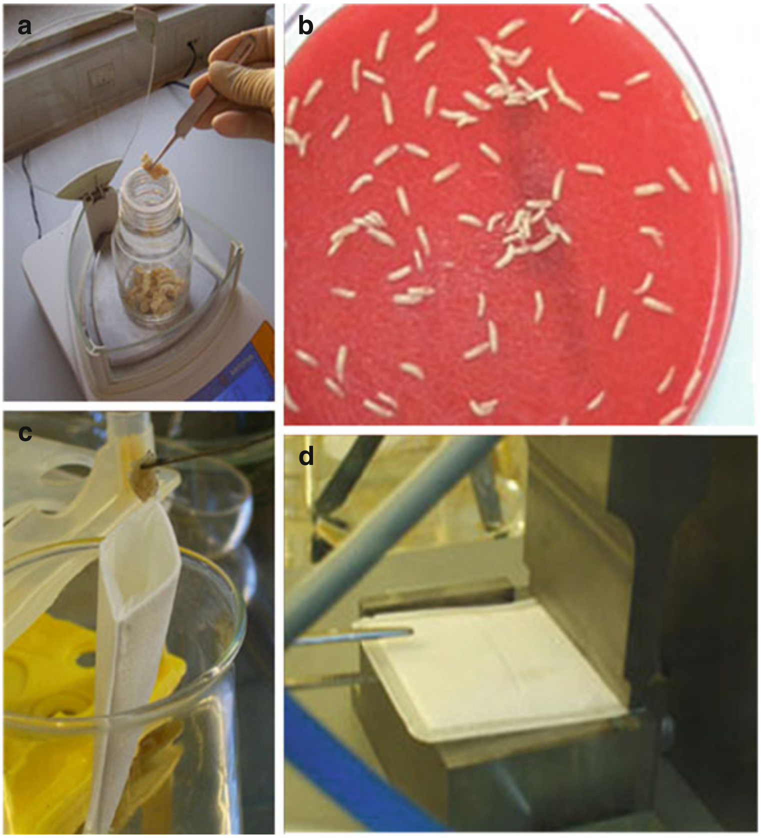


Fig. 4.1 Production of biobags with *Lucilia sericata* maggots at Biomonde: **(a)** Inoculation with a defined number of eggs, **(b)** growth on sterile agar, **(c)** filling of bags with maggots, and **(d)** closure of the bags (Pictures were kindly provided by BioMonde Laboratories®)

Ultrafiltration of secretions allowed the detection of at least two anti-bacterial factors. The smaller factor was present in the fraction containing compounds with molecular masses below 500 Da and exhibited lytic and anti-MRSA activity corresponding to that in native secretions, while the larger one, determined in the 0.5–3-kDa fraction, was active against non-methicillin-resistant *S. aureus* (Bexfield et al., 2004).

The first anti-bacterial substances purified from hemolymph extracts of *L. sericata* were isolated using high-performance liquid chromatography and subsequently characterized by gas-chromatography coupled with mass spectrometry which resulted in identification of three compounds: p-hydroxybenzoic acid (138 Da), p-hydroxyphenylacetic acid (152 Da), and octahydro-dipyrolo pyrazine-5,10-dione (194 Da), also known as the cyclic dimer of proline, proline diketopiperazine or cyclo (Huberman et al., 2007). All compounds exhibited in vitro

activity against *Micrococcus luteus* and/or *P. aeruginosa*, particularly when tested in combination, implicating synergistic functions in microbial killing.

The first genes contributing to anti-microbial defense of *L. sericata* maggots have recently been identified using suppression subtractive hybridization (Altincicek and Vilcinskas, 2009). This PCR-based method amplifies differentially expressed cDNAs while suppressing amplification of common cDNAs, and has been proven to be a powerful tool in targeted screening for genes that are induced upon challenge with microbial elicitors of innate immune responses such as bacterial lipopolysaccharide (LPS). This approach has been successfully applied to identify immunity-related genes in a number of insect species for which no genome sequence is available such as the ancient apterygote firebrat *Thermobia domestic*a (Altincicek and Vilcinskas, 2007a) or the rat-tailed maggots of the drone fly *Eristalis tenax* which are the only animals capable of surviving in aquatic habitats with extreme microbial contamination such as farmyard liquid manure storage pits (Altincicek and Vilcinskas, 2007b). Application of the suppression subtractive hybridization method in targeted screening for immunity-related molecules in *L. sericata* resulted in identification of 65 genes that are induced upon challenge with LPS, among which numerous putative anti-microbial peptides and proteins as well as digestive enzymes have been found (Altincicek and Vilcinskas, 2009).

A diptericin-like anti-microbial peptide has been discovered among the immune-inducible genes. Phylogenetic analysis elucidated that the diptericin from *Lucilia* is more similar to homologs from Muscidae (*Musca domestica* and *Stomoxys calcitrans*) than to diptericins from flies belonging to the Drosophilidae. Comparative analysis with related diptericin-like sequences from *Anopheles gambiae* and *Culex pipiens* belonging to the Culicidae, which were used as an out-group, resulted in a phylogenetic tree similar to that generated using mitochondrial or ribosomal genes, suggesting neither positive nor negative selection of diptericin genes among Diptera (Altincicek and Vilcinskas, 2009). In contrast, three open reading frames of putative proline-rich anti-microbial peptides have been found which contain predicted signal sequences for extra-cellular secretion, and which share only low similarity with proline-rich anti-microbial peptides known from other dipterans such as drosocin and metchnikowin from *Drosophila*. Synthetic analogues of these Lucilia proline-rich anti-microbial peptides have been proven to exhibit activity against Gram-positive *M. luteus*, but not against Gram-negative *Escherichia coli* (Altincicek and Vilcinskas, 2009).

The immunity-related transcriptome of *L. sericata* encompasses peptides sharing similarity with the N-terminal part of sapecin-B (an insect-type defensin) (Altincicek and Vilcinskas, 2009). Confirming this finding, a sapecin-like defensin consisting of 40 residues and stabilized by three intra-molecular disulfide bonds has recently been purified from extracts from gut, salivary glands, fat body, and hemolymph, which was named Lucifensin (Cerovsky et al., 2010). Another identified component up-regulated upon immune-stimulation in *L. sericata* is lysozyme, which is well-known for its ability to hydrolyze the sugar linkages within the murein sacculus of Gram-positive bacteria, and which also contributes to antifungal defense in insects (Vilcinskas and Götz, 1999). The lysozyme gene found in

L. sericata shares the highest similarity with that of *M. domestica* and this allowed, therefore, one to calculate a three-dimensional model of the *L. sericata* lysozyme using the published crystallographic structure of the *M. domestica* lysozyme as a template. Both lysozymes differ significantly in a particularly exposed loop, which may reflect adaptation to different targets (Altincicek and Vilcinskas, 2009).

4.8 Inducible Digestive Enzymes

The experimental approach mentioned above enabled also the identification of a number of potential digestive enzymes among which some could be involved in tissue debridement. Degradation of extra-cellular matrix components by defined proteinases secreted by the maggots has been documented by Chambers et al. (2003). Targeted screening for inducible digestive enzymes by suppression subtractive hybridization resulted in identification of two lipases and nine trypsin-like serine proteinases. In addition, at least two cysteine proteinases, as well as one aspartatic and one metalloproteinase are induced upon microbial challenge. The latter belongs to the M14 family of metalloproteinases, whose functions have been attributed to digestion of food, processing of bioactive peptides, or the hydrolysis of bacterial cell walls (Rawlings and Morton, 2008). However, the precise function of these enzymes and their potential contribution to the beneficial effects of *L. sericata* secretions to wound healing remain to be elucidated.

4.9 Future Directions

Presently, we envisage a switch from traditional maggot therapy to modern biosurgery. The latter can be defined as maggot therapy without maggots, but with derivatives or molecules thereof. Several research groups worldwide have started to isolate and characterize the beneficial molecules within the secretions of medicinal maggots such as enzymes mediating debridement. The aim is to produce defined molecules with therapeutic value either synthetically or by recombinant gene expression. Artificial production of maggot-derived compounds would allow direct application in wound therapy using novel dressings impregnated with hydrogels as carrier substrates. Unlike living maggots, maggot-derived therapeutics should not be negatively perceived by patients, opening the possibility to expand their use beyond wound healing to include the therapy of other diseases.

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Chapter 5
Insect-Associated Microorganisms as a Source for Novel Secondary Metabolites with Therapeutic Potential

Helge B. Bode

Abstract Natural products play an essential role in our everyday life as almost all antibiotics or anti-cancer compounds currently in clinical use are either natural products or derivatives thereof. Mainly due to increasing resistance against these antibiotics there is an urgent need for novel bioactive natural products and several strategies are currently in use to find new compounds. In this chapter insects are suggested as a new and very promising source for novel secondary metabolite-producing bacteria and fungi. Whereas entomopathogenic fungi have been known for quite some time as potent producers of different bioactive compounds and have been used in traditional Chinese medicine for more than 2000 years, almost nothing is known about the underlying biochemistry and molecular biology that is involved in the biosynthesis of such compounds. Similarly, entomopathogenic bacteria have only been proven to be a rich source of interesting compounds during the last 20 years and recent genome sequencing projects have revealed their great potential as secondary metabolite producers. Furthermore, bacteria that live in symbiosis with insects have also been shown to be a rich source of potent natural products which have to be explored in the future in more detail.

Keywords Secondary metabolites · Entomopathogenic and insect-associated microorganisms · Drug discovery · Bacteria · Fungi

5.1 Introduction

Without natural products namely secondary metabolites our life would be less colourful as most flower pigments are in fact secondary metabolites. More importantly, it would be less comfortable and definitely much shorter as more than 75%

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of all antibacterial and approximately 50% of all anti-cancer compounds currently in clinical use are either natural products or derivatives thereof (Newman and Cragg, 2007). Especially in industrialized countries we have become so familiar with using antibiotics to treat bacterial infections that almost everybody has taken them at least once in his/her life. However, the times of the "magic bullets" as they were called by Paul Ehrlich almost 100 years ago seem to be over: Presently we have resistance even against our last resort antibiotics and also against very recently introduced new antibiotics. This has resulted in the return of almost eradicated diseases (e. g., tuberculosis) even in the industrialized countries. However, no matter how responsible we will be with new antibiotics, we can never avoid resistance, simply due to the speed by which bacteria grow, mutate and exchange their genes. This global problem will even increase in the future as almost no new antibiotics are currently being developed by the pharmaceutical industry because it is very time consuming and expensive compared to the development of "life-style" drugs or pharmaceuticals to treat chronic diseases (von Nussbaum et al., 2006).

What can we do? How can we at least maintain the current status-quo? How can we avoid pre-antibiotic times with people dying from "simple" infectious diseases? Since natural products have a much higher chance of being a promising antibiotic or anti-cancer compound compared to synthetic chemicals, one idea might be to revive natural product research as described recently (Bode and Müller, 2005). Here one possibility might be to find and explore new sources for natural products like rare soil bacteria or marine organisms. Clearly, the identification of new compounds would just be a matter of statistics as the whole drug discovery process is mostly random and hardly predictable and a more targeted approach would be much more desirable. This chapter will introduce the use of insect pathogenic and insect-associated microorganisms as (1) a new and powerful source for new and promising natural products and (2) will also highlight the analysis of insect-associated microorganisms as a more targeted approach to finding such compounds.

Why insect-associated microorganisms? Insects are the most diverse group of animals on our planet (Chapman, 2007). Insects are also a very old group of animals and since their first appearance almost 400 million years ago they have adapted to several different environments and therefore are ubiquitous and can be found almost everywhere (except for the oceans). Therefore, it is not surprising that several symbiotic and/or pathogenic microorganisms have been adapted specifically to insects as the host system. As microorganisms are usually genetically tractable, insects can be regarded as a huge reservoir for biotechnologically and pharmaceutically important microbes. A well-known example, which has been explored in detail are termites and their gut bacteria, which are able to degrade wood and actually allow the termites to destroy everything made from wood including complete houses. On the other hand, the responsible enzymes, microorganisms and/or pathways would be very useful in the textile industry (cotton treatment) or for biofuel production (for the degradation of all kinds of plant material) and there is currently a large interest in such microorganisms (Matsui et al., 2009).

The rationale behind the idea of using insect-associated microorganisms as a source for new bioactive natural products is the following: As insects live in the same environment as humans, they are also challenged by the same or closely related pathogens as humans. This is especially important since insects and mammals in general share a large portion of their physiology including parts of the immune system. Therefore, insects can also be used as model systems for different human infectious diseases (see Part I, Chapter 1) (Mylonakis et al., 2007; Mylonakis, 2008; Champion et al., 2009; Vilcinskas, 2009). Accordingly, even the analysis of entomopathogenic microorganisms makes sense since toxic compounds that can kill insects might also be potent anti-cancer compounds.

5.2 Entomopathogenic Fungi

Approximately 80,000 fungi are currently known, which might represent about 5% of all fungi on our planet (Hawksworth and Rossman, 1997). Of these also a small fraction is known which live in association with insects: Some are commensals which derive nutrients from the insect gut or from the insect cuticle without causing any harm to the host. However, some are also truly pathogenic and derive nutrients directly from the insect (Samson et al., 1988). Entomopathogenic fungi produce extra-cellular lipases, chitinases and proteases which help them to overcome the insect cuticle as the first barrier of infection. Once inside the insect, the fungus develops as a yeast-like form and produces secondary metabolites that inhibit the insect immune system, modify the insect's behaviour, or act as post-mortem antibiotics against competing microorganisms like other fungi or bacteria. Therefore, entomopathogenic fungi are receiving increasing attention and are regarded as promising sources of novel bioactivities not only for antibiotics but also for diseases increasing in prevalence like Alzheimer's disease.

The best-known entomopathogenic fungi are members of the highly host-specific metagenus *Cordyceps* that consists of more than 300 species (Hywel-Jones, 2002). Besides the sexual state (*Cordyceps*) several asexual states of these fungi are known, including well-known secondary metabolite producers exemplified by *Metarhizium*, *Beauveria* or *Hirsutella*. *Cordyceps sinensis* has been known for about 2000 years and has been used in Chinese traditional medicine. However, since the demand for alternative medicines has spread in Western culture it almost became a life-style drug in some societies. Although only a few scientifically valid studies have been published about the benefit of *C. sinensis*, it has been harvested to an extent that it is currently an endangered species (Paterson, 2008). Natural product research during the last years has led to the identification of several bioactive secondary metabolites, which have been evaluated pharmaceutically as potential lead compounds and which help to understand the benefits described in traditional Chinese medicine. As recent publications have reviewed the natural products isolated from *Cordyceps* and its asexual states (Paterson, 2008; Isaka et al., 2005), this chapter will only highlight a few very recent and/or bioactive examples as well as new ways to even increase the number of compounds from these fungi (Fig. 5.1).

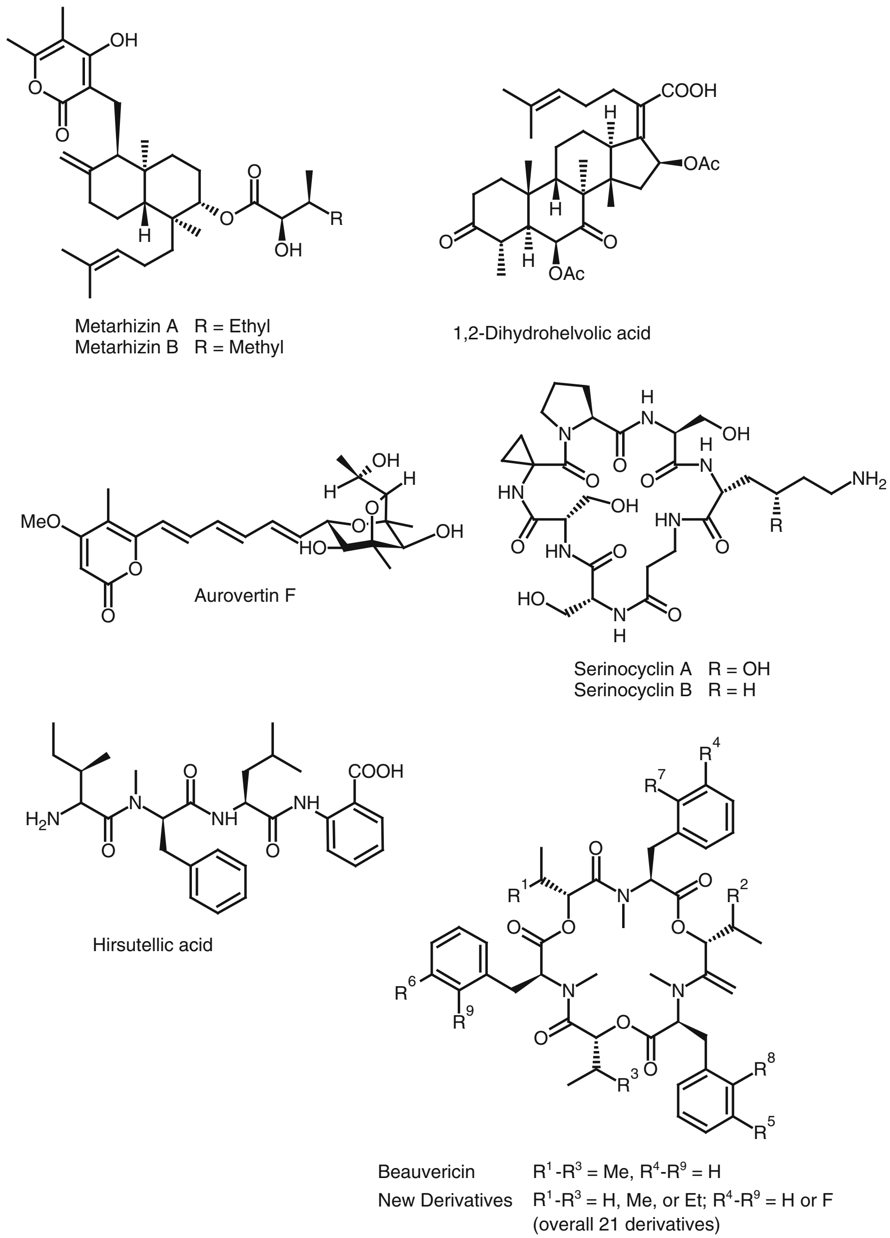


Fig. 5.1 Selected natural products recently isolated from entomopathogenic fungi

The new diterpene pyrone compounds metarhizin A and B have been isolated from *Metarhizium flavoviride* and they showed potent and selective anti-proliferative activity against both insect and human cancer cell lines, which would make them novel lead structures for anti-cancer agents and probes for cell cycle regulation (Kikuchi et al., 2009). From *M. anisopliae* the new compound 1,2-dihydrohelvolic acid, which showed good antibiotic activity against *S. aureus*, has been identified from cultures grown in medium with the insect component as the sole carbon and energy source (Lee et al., 2008). From other *M. anisoplia*e strains the new aurovertins F-H (Azumi et al., 2008) as well as serinocyclins A and B (Krasnoff et al., 2007) have been isolated. Serinocyclin A and B are unusual members of the fungal cyclopeptides as they show no *N*-methylation and therefore are quite polar. Serinocyclin A produced a sub-lethal locomotory defect in mosquito larvae at an EC_{50} of 59 ppm. Hirsutellic acid A has been isolated from a *Hirsutella* species and showed inhibition of *Plasmodium falciparum* with an IC_{50} value of 8.0 μM while it was essentially nontoxic to Vero cells (Thongtan et al., 2006).

New non-natural cytotoxic and anti-haptotactic beauvericin analogues have been obtained from precursor-directed biosynthesis of *Beauveria bassiana* (Xu et al., 2007). In the precursor-directed biosynthesis non-natural precursors similar to the natural precursors are fed to the producing culture (Bode et al., 2002). Incorporation of these artificial building blocks into the desired compounds leads to the formation of new derivatives, which might have superior biological activity as described previously for several classes of compounds. In order to overcome the general problem of precursor-directed biosynthesis, which is the additional production of the natural compound resulting in a yield reduction of the desired derivatives, one can use a mutasynthesis approach (Weissman, 2007; Weist and Süssmuth, 2005). Here the gene(s) encoding the biosynthesis of the precursor that should be substituted against an artificial one are mutated resulting in non-producing strains which can be complemented by the addition of the natural or artificial precursors which then would result in the production of the natural product or of non-natural derivatives thereof, respectively. The basis of such an approach is always the knowledge of the biosynthesis gene cluster involved in the respective biosynthesis. For *B. bassiana* this was pioneered by the group of Istvan Molnár who identified the biosynthesis gene clusters for the production of beauvericin and bassianolide in *B. bassiana* (Xu et al., 2009a; 2008). This enabled them to perform mutasynthesis via disruption of the ketoisovalerate reductase and complementation of the resulting loss of D-2-hydroxyisovalerate with D-2-hydroxybutyrate or D-2-hydroxy-3-methylvalerate. Parallel addition of fluorinated phenylalanine derivatives resulted in the production of overall 16 new beauvericin derivatives with in some cases better biological activity than the natural beauvericin (Xu et al., 2009b).

5.3 Entomopathogenic Bacteria

Entomopathogenic bacteria can be regarded as a novel source of potential pharmaceuticals, which were explored only recently. Additionally, other entomopathogenic bacteria have been identified which have not been analyzed for secondary

metabolites and/or genes encoding the corresponding biosynthesis genes but are members of well-known secondary metabolite-producing genera (Vallet-Gely et al., 2008). Examples are *Serratia entomophila* (Grimont et al., 1988) and *Erwinia carotovora* (Basset et al., 2000). Whereas the entomopathogenic activity of *E. carotovora* has been shown under lab conditions only, *S. entomophila* has been isolated from the New Zealand grass grubb *Costelytra zealandica* in the field. *S. entomophila* causes the so-called amber disease in *C. zealandica*, a major pasture pest in New Zealand.

The best known example of an entomopathogenic bacterium is *Bacillus thuringiensis* which produces parasporal protein crystals in association with spore formation. These protein toxins lyse the insect after ingestion (Zhou et al., 2008). Therefore, natural and genetically engineered strains of *B. thuringiensis* have been used commercially to control pests of many representatives of the orders of *Lepidoptera*, *Diptera* and *Coleoptera*. Additionally, the genes encoding the Bt-toxins have been successfully transferred into cotton, corn, soybean and rice conferring resistance to insect pests thus leading to a significant economic benefit. This technology is considered to be one of the most successful models in agricultural biotechnology. The advantage of such crops is that fewer insecticides have to be applied to the fields but it is not clear how these genetically modified crops behave in nature over time and influence beneficial insects or insect diversity in general. Moreover, specific pests have become resistant against specific Bt-forms resulting in an arms race between agricultural biotechnology and insects similar to the arms race between the pharmaceutical industry and multi-resistant bacteria. Besides protein toxins *B. thuringiensis* is also a producer of an acyl homoserine lactone lactonase which can quench pathogenicity of plant pathogenic bacteria and zwittermicin A (Fig. 5.2), a potent antibiotic and anti-fungal compound (Zhou et al., 2008). Zwittermicin A is a linear aminopolyol antibiotic, which represents a new class of antibiotics as its biosynthesis is very unusual (Kevany et al., 2009). Recent work has shown that zwittermicin acts synergistically with the Bt-toxin which would make it very useful in agricultural biotechnology as well (Zhou et al., 2008). Its natural function might be to protect the Bt-toxin killed insect cadaver from opportunistic food competitors like other soil-living bacteria and fungi (as in the case of *Photorhabdus* and *Xenorhabdus*; see below). One can speculate that additional natural products might be produced by *B. thuringiensis* since *Bacillus* in general is a rich source of secondary metabolites (Chen et al., 2009). Indeed, BLAST searches using PKS or NRPS enzymes as bait reveal several additional enzymes/biosynthesis gene clusters in sequenced *B. thuringiensis* strains (Challacombe et al., 2007; Han et al., 2006) whose corresponding structures have not been identified yet.

Similar to *B. thuringiensis*, *Pseudomonas entomophila* is a soil bacterium that, after ingestion, kills insects of different orders (Vodovar et al., 2006). Genetically it shows great similarity to the closely related strain *P. putida* KT2440 but harbours several genes encoding putative insecticidal toxins, proteases and hemolysins as well as biosynthesis gene clusters for the production of hydrogen cyanide and novel secondary metabolite gene clusters. Although no secondary metabolite has been identified from *P. entomophila*, analysis of the biosynthesis gene clusters

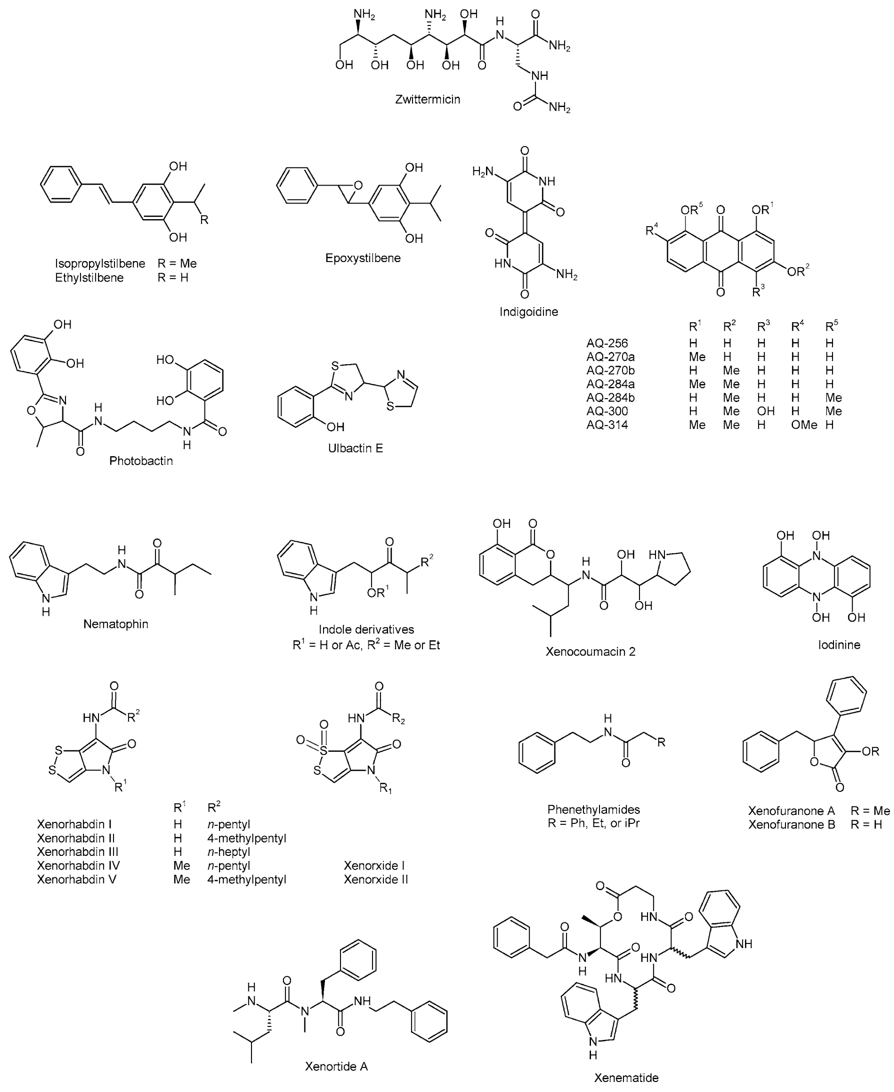


Fig. 5.2 Known natural products from entomopathogenic bacteria

might indicate the biosynthesis of pyoverdine derivatives that are typical to fluorescent pseudomonads as well as a siderophore with similarity to acinetobactin from *Acinetobacter baumannii* (Mihara et al., 2004). Additionally, biosynthesis gene clusters for the production of three different lipopeptides and a polyketide have been identified in the genome (Vodovar et al., 2006).

Probably the best studied examples of entomopathogenic bacteria are *Xenorhabdus* and *Photorhabdus*, members of the family *Enterobacteriaceae*, which engage in a mutualistic association with the entomopathogenic nematode *Steinernema* or *Heterorhabditis*, respectively, and are also pathogenic towards

different insect hosts (Goodrich-Blair and Clarke, 2007; Herbert and Goodrich-Blair, 2007). The bacteria are carried by the infective juvenile (IJ) stage of the nematode, which infects the insect host by penetrating through natural openings such as the mouth, anus, or spiracles. The bacteria are released from the nematode into the insect's body cavity (hemocoel) and both kill the host rapidly within 48 h. The nematode subsequently reproduces for several generations in the hemocoel feeding on both the bacteria and the nutrients derived from insect sources. With the depletion of nutrient supplies the nematodes develop into the IJ stage acquiring its bacterial partner from the hemocoel, before emerging from the insect cadaver into the soil and searching for new insect hosts. This complex life cycle that involves mutualistic as well as pathogenic interactions make *Photorhabdus* and *Xenorhabdus* an ideal model to study the differences and similarities of symbiosis and pathogenesis (Goodrich-Blair and Clarke, 2007).

During the complex life cycle the bacteria not only have to kill the insect host using several protein toxins but also have to defeat several other microbes that are direct food competitors: Saprophytic microbes from the soil as well as bacteria adhering to the insect gut or cuticle of the nematode represent other potential sources of competitors that can grow within the insect cadaver. It was recently shown that bacteria present in the insect gut appeared in the hemolymph soon after nematode invasion (Gouge and Snyder, 2006). These competitors were eliminated as *X. nematophila* became the dominant microbial species in the hemolymph. To successfully compete for nutrient resources of the insect *Xenorhabdus* and *Photorhabdus* produce phage-derived bacteriocins (Thaler et al., 1995), colicin E3-type killer proteins (Singh and Banerjee, 2008), and insect toxin complexes (ffrench-Constant and Waterfield, 2006) as well as several secondary metabolites (Piel, 2004; 2009).

Photorhabdus luminescens ssp. *laumondii* strain TT01 was the first completely sequenced member of this group of entomopathogenic and nematode-associated bacteria (Duchaud et al., 2003). Within the genome sequence several insecticidal proteins, hydrolytic enzymes (proteases, lipases, chitinases) have been identified which are involved in the complex life cycle between the bacterium, its nematode host *Heterorhabditis*, as well as the insect prey. Moreover, several genes encoding enzymes involved in secondary metabolite biosynthesis have been identified and a careful analysis of the genome sequence led to the identification of at least 23 biosynthesis gene clusters that correspond to more than 6.5% of the overall genome sequence (unpublished data). Thus, with respect to secondary metabolism *Photorhabdus* is very close to the Gram-positive bacteria of the genus *Streptomyces* that are usually regarded as potent secondary metabolite producers. Most of the identified biosynthesis gene clusters encode non-ribosomal peptide synthetases (NRPS) (Sieber and Marahiel, 2005) that are involved in the biosynthesis of linear or cyclized peptides, lipo- or depsipeptides and two encode hybrids of polyketide synthases (PKS) (Van Lanen and Shen, 2008; Staunton and Weissman, 2001) and NRPS. Moreover, one biosynthesis gene cluster encoding an unusual fatty acid synthase (FAS) or a FAS/PKS hybrid, a type II PKS, as well as two biosynthesis gene clusters involved in siderophore biosynthesis have been identified.

A similar richness in secondary metabolite biosynthesis gene clusters with a similar concentration of NRPS has been identified in *Photorhabdus asymbiotica* which is a human pathogenic member of the genus *Photorhabdus* (Waterfield et al., 2008).

Contrary to this rich diversity, *Photorhabdus* was only known to produce isopropyl- and ethylstilbenes (Hu et al., 2006), anthraquinones (AQ) (Li et al., 1995b) and the siderophore photobactin (Ciche et al., 2003) (Fig. 5.2). Besides the biosynthesis gene clusters for the production of these three compounds (Ciche et al., 2003; Brachmann et al., 2007; Joyce et al., 2008), the biosynthesis gene clusters for the production of carbapenem (Derzelle et al., 2002) have been identified. Moreover, several peptides including cytotoxic pentapeptides and yersiniabactin derivatives (e.g., ulbactin E) could be identified after detailed analysis of the crude extracts or after heterologous expression of the respective biosynthesis gene clusters (Fig. 5.2) (Waterfield et al., 2008). Heterologous expression also led to the identification of the NRPS-derived blue pigment indigoidine (Brachmann and Bode, unpublished) already known from *Erwinia* and *Streptomyces* (Reverchon et al., 2002; Takahashi et al., 2007).

Especially AQ and stilbenes are very simple compounds that nevertheless serve complex ecological roles and are the result of unusual biosyntheses: AQ are common metabolites of plants, fungi and bacteria. They are also postulated to serve an important ecological function in *Photorhabdus* as this class of compounds in general is known as an ant and bird deterrent (Pankewitz and Hilker, 2008) that would ensure that infected larvae avoid potential predation by ants and birds. Biochemically, AQ in *Photorhabdus* are formed following a type II PKS mechanism which is only the second example of a type II PKS biosynthesis gene cluster from Gram-negative bacteria (Brachmann et al., 2007).

Stilbenes are typical plant metabolites (e.g., resveratrol in grapes and red wine) and *Photorhabdus* is the only stilbene producer outside the plant kingdom. Moreover, stilbene biosynthesis in *Photorhabdus* is different than the plant biosynthesis as the final step includes a head-to-head condensation of two β-ketoacyl intermediates instead of the consecutive elongation of cinnamoyl-CoA by malonyl-CoA in the plant pathway (Joyce et al., 2008). Isopropylstilbene shows antibiotic activity against Gram-positive bacteria and fungi (Eleftherianos et al., 2007), inhibits the phenol oxidase that is part of the insect immune system (Eleftherianos et al., 2007) and is required as a signal molecule for proper nematode development (Joyce et al., 2008). Therefore, it is truly multifunctional and has been identified in all *Photorhabdus* strains analyzed so far. Thus, it can be regarded as the "Swiss army knife" of *Photorhabdus* as it is involved in almost all different parts of the complex tritrophic relationship between bacteria, nematodes and insects.

The genomes of *Xenorhabdus nematophila* and *Xenorhabdus bovienii* have been sequenced during a NSF-funded project and revealed several biosynthesis gene clusters involved in the biosynthesis of secondary metabolites. Compounds described in the literature represent mostly small molecules like benzylideneacetone (Ji et al., 2004), iodinine (Fodor et al., 2008), phenethylamides and indole derivatives (Li et al., 1995a; McInerney et al., 1991a) but also more complex compounds like

the xenorhabdins and xenorxides (Li et al., 1998), and xenocoumacins (McInerney et al., 1991b) derived from hybrid PKS/NRPS systems are known (Fig. 5.2). Despite their simple structure, several different biological activities have been described for most of these compounds and their biosynthesis genes have not been identified yet except for the xenocoumacins, which are derived from a PKS-NRPS hybrid system (Reimer et al., 2009).

Recently xenematide and xenortides have been identified as the first peptides from *X. nematophila* with xenematide showing weak insecticidal activity (Lang et al., 2008). Biochemically, these compounds are derived from NRPS enzymes and indeed the corresponding biosynthesis genes have been identified in our group (Reimer and Bode, unpublished).

5.4 Bacteria as Insect Symbionts

In the previous chapters several entomopathogenic fungi and bacteria have been described, which are also potent producers of interesting secondary metabolites. However, insects in general are also a rich source of microorganisms. Taking into account the vast number of insect species it is not surprising that interesting secondary metabolite producers are among these insect-associated microorganisms. This chapter is not dedicated to secondary metabolites isolated from insects although some of these metabolites might in fact be derived from associated bacteria and/or fungi as also found in the sponge–bacteria association in the oceans (Bode and Müller, 2005). The focus of this chapter are recent results from insect–bacteria symbioses, which were probably established several million years ago and which help us to understand the function of the secondary metabolites involved as well as such important issues like strategies in drug discovery as well as resistance development against clinically used antibiotics as described below (Fig. 5.3).

Already in 2002 Jörn Piel could identify a polyketide synthase-peptide synthetase encoding biosynthesis gene cluster from an uncultured bacterial symbiont of *Paederus fuscipes* beetles (Piel, 2002). These beetles have been known for a long time for the production of pederin, a highly cytotoxic compound which also shows good anti-cancer activity. With the isolation of the corresponding biosynthesis gene cluster Piel could prove that a bacterium from the genus *Pseudomonas* is the actual producer. Moreover, this groundbreaking work also has implications for the sustainable production of this and similar compounds as heterologous production in suitable hosts now becomes a realistic scenario. Unfortunately, nothing is known about the symbiosis between the beetle and the symbiont. However, from the fact that the symbiont can not be cultivated as other free-living Pseudomonads, one can conclude that the symbiosis is quite tight.

A more complex example of an insect–bacteria symbiosis was described in 2005 by Kaltenpoth et al. (Kaltenpoth et al., 2005). They analyzed the European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae), a solitary digger wasp that constructs nest burrows in sandy soil. Beewolf females catch and paralyze honeybees and use them as a food source for their larvae in these soil nests. The larvae feed on the bees and spin a cocoon in which they hibernate, until the new

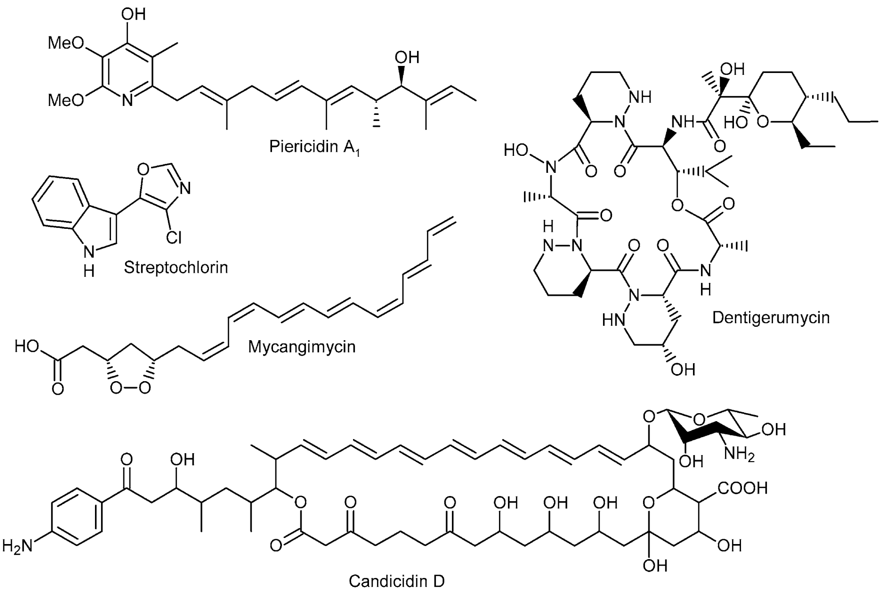


Fig. 5.3 Natural products recently identified from bacterial insect symbionts

generation of beewolfs emerges the subsequent summer. The brood cell is humid and warm, ideal conditions not only for the larvae but also for bacteria and fungi that live in the soil and thus could infect and kill the larvae. How are the larvae protected from infections during this long period of time? The answer to this question is that beewolf females smear a white substance from their antennae against the ceiling of the finished brood cell. This white substance are *Streptomyces* bacteria (Kaltenpoth et al., 2006), which are postulated to produce antibiotics and which are "cultivated" for this purpose in specialized antennal glands. The deposition of the bacteria dramatically enhances the survival of the larvae and also protects the cocoon as the bacteria could also be found in the cotton silk. Very recently the natural products produced by this bacterium were identified as the known antibiotics streptochlorin and different piericidin derivatives (Kroiss et al., 2010) (Fig. 5.3).

Even more complex examples come from the groups of Currie and Clardy who investigated the southern pine beetle (*Dendroctonus frontalis*) and leaf cutting ants (see below). The southern pine beetle can kill healthy pine trees via pheromone-guided mass attacks and thus are also an economically important pine tree pest (Hofstetter et al., 2006). The beetles live in symbiosis with the fungus *Entomocorticium* sp. A, which serves as a food source for the beetle larvae. Adult beetles carry the fungus in a specialized storage compartment called the mycangium and during excavation of ovipositional galleries within the inner bark of the tree they inoculate these galleries with the fungus. However, the antagonistic fungus *Ophiostoma minus*, which lives on *Tarsonemus* mites that themselves live on the beetle, can outcompete *Entomocorticium* sp. A and thereby disrupt larval development. To maintain the *D. frontalis*–*Entomocorticium* sp. A symbiosis, the

beetles additionally carry a *Streptomyces* sp. in their mycangium, which shows high similarity to *S. thermosacchari*. Thus, the galleries are inoculated with the nutrient source and its protecting agent. The groups of Currie and Clardy could isolate a natural product named mycangimycin from the bacterium which they could grow in standard *Streptomyces* medium (Scott et al., 2008). Mycangimycin was shown to efficiently inhibit *O. minus* while only slightly affecting *Entomocorticium* sp. A. It also inhibits human pathogenic *Candida albicans* including amphotericin-resistant mutants (Oh et al., 2009b). The 1,2-dioxolane functionality of mycangimycin is similar to pharmacophores with anti-malarial activity and thus it showed also activity against *P. falciparum* similar to clinically used anti-malarial drugs (Oh et al., 2009b). Clearly the next steps could be the identification of the mode-of-action in fungi and protozoa as well as the generation of simplified and more stable analogues.

A second example from the groups of Currie and Clardy is the highly evolved and very old symbiosis between fungus-growing ants and their fungi (Currie, 2001). The probably best studied system here is the one between leaf-cutter ants and their fungal cultivars. The ants carefully tend the fungus, which serves as their major food source, feed it with fresh leaves, and protect the fungal garden from microbial pathogens including the specialized and highly virulent and devastating fungi of the genus *Escovopsis*. So the question arises how the cultivar monoculture is maintained and protected: Again, actinobacteria but also other bacteria (Santos et al., 2004) have been identified from the ant surface which produce anti-fungal activities. Already in 1999 Currie et al. identified a *Pseudonocardia* strain that could efficiently inhibit the growth of *Escovopsis* (Currie et al., 1999), and very recently they could identify the responsible compound together with the Clardy group. Only a single compound was produced by the *Pseudonocardia* strain, which turned out to be a structurally and biochemically interesting new cyclic depsipeptide which they named dentigerumycin (Oh et al., 2009a). It consists of several unusual amino acids and has a complex polyketide-derived side chain. Bioassays revealed that dentigerumycin clearly inhibits the pathogenic *Escovopsis* while the cultivar was resistant. Similarly to mycangimycin, *C. albicans* including amphotericin-resistant mutants were also inhibited by dentigerumycin.

Additionally, the group of Spiteller isolated *Streptomyces* sp. with similarity to *S. albidoflavus* or *S. griseus* from several different leaf-cutting ants and could show that they produce the macrolide candicidin D and additional candicidin derivatives (Haeder et al., 2009). At least one associated microorganism in all three leaf-cutting ant species analyzed was shown to produce these known anti-fungal compounds. Candicidins also showed very good activity against *Escovopsis* but was either not or only weakly active against other pathogenic fungi. As expected, the authors could also identify candicidin biosynthesis genes in the candicidin-producing *Streptomyces* strains. Candicidins are clinically used and highly active antifungals due to their interaction with sterols in the fungal cell membrane leading to K^+-leakage and subsequent cell death. As resistance against polyene macrolides has been rarely observed the authors suggest that the leaf-cutting ants use a similar strategy as humans in that *Escovopsis* can not adapt quickly enough to this type of compound.

5.5 Conclusions

Why are there not more compounds known from entomopathogenic bacteria or fungi or from insect symbionts? The answer is simply because we have only recently started to look into this new and promising source but soil-living bacteria like *Streptomyces* have been studied in detail during the last 60 years.

Thailand has a very rich biodiversity and thus also seems to be a good place to look for entomopathogenic fungi as several new strains have been isolated there during the last years (Isaka et al., 2005). However, there are other countries with a similarly high biodiversity that have not been studied in detail for such fungi.

Taking into account the large number of different bark beetles or other fungi-growing ants one can clearly expect the compounds described above to show up in some strains. However, there is also the likely chance of finding new and different compounds from closely related biological systems as clearly shown by the identification of the structurally totally different compounds dentigerumycin and candicidin from leaf-cutting ants.

A significant problem of natural product research is the random nature by which this research and drug discovery in general is usually performed. Different compounds are isolated from different sources and tested against different targets in order to find a useful activity. Especially for anti-infective research the use of insects as a resource for bacteria and fungi producing pharmaceutically interesting compounds would be a much more rational approach: As mentioned in the Introduction of this chapter and in more depth in Part I, Chapter 1, insects are quite similar to humans and therefore encounter similar pathogenic organisms. Thus similar anti-infective compounds might be used for the treatment of these pathogens.

In summary, several new natural products will be identified in the future from insect-associated or entomopathogenic microorganisms if we are willing to explore this promising new source for bioactive natural compounds. The time to do so has never been better as we now have all analytical (e.g., sensitive and affordable mass spectrometers) and molecular tools (e.g., affordable and fast whole-genome sequencing) available. Clearly natural product research and drug discovery in general will benefit from insects in the future.

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Chapter 6
Potential Pharmaceuticals from Insects and Their Co-Occurring Microorganisms

Konrad Dettner

Abstract Because of their enormous species diversity insects represent an interesting and promising source for low molecular biologically active natural products which either are de novo synthesized by the insect or by associated microorganisms. Many of the structures show that potential pharmaceuticals can be found which may be used for human and veterinary medicine. Concerning the toxic terpene anhydride cantharidin it is demonstrated that synthetically obtained low toxic analogues may be suitable for use as pharmaceuticals or in other contexts. In addition low molecular compounds from insect-derived microorganisms are compiled according to the taxonomy, this means the order of the host insects as far as these compounds are of pharmaceutical interest. Remarkably various compounds have also been described from other non-insect sources. It is shown that only a few natural products, such as pederin, result from a true symbiotic interaction between host insect and bacteria. In most other cases the presented metabolites from insect-derived microorganisms are produced in the lab and it has to be clarified whether they are even produced within the host insect.

Keywords Secondary compounds · Cantharidin · Insect-symbionts · Symbiotic fungi and bacteria · Insects · Bioprospecting · Pharmaceuticals

6.1 Introduction

The systematic search for natural compounds in nature with a potential for product development into pharmaceuticals, pesticides, cosmetics, or food additives by biochemical methods without disruption to nature is called chemical prospecting within a discipline entitled bioprospecting (Mateo et al., 2001). Among the best-selling pharmaceuticals in 1997, 13 were derived from or developed as the result of leads generated by natural products from plants, animals, and microorganisms.

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There were three products derived from natural compounds from animals: The acetylcholinesterase-inhibitors Vasotec (Merck & Co.) and Zestril (Zeneca) were developed from a peptide in a snake venom (*Bothrops*), whereas the anti-viral drug Zovirax (Glaxo Wellcome) was derived from a cytosine arabinoside isolated from a Floridan sponge (Mateo et al., 2001). In addition Newman and Cragg report in 2007 that a significant number of natural products or leads are actually produced by microbes or by microbial interaction with the host from whence it was isolated.

Because of the enormous species diversity of approximately 10 million organismic species (Dettner and Peters, 2003), the described insects (more than 1 million species) and other arthropods such as myriapods (more than 15,000 species), arachnids (more than 93,000 species), and crustaceans (about 43,000 species) represent a true chemical treasure for future generations (see Eisner, 2003; Eisner et al., 2005; Dettner, 2010). Apart from the ocean, insects are found on land and in freshwater, where about 80% of all organisms have been described. Therefore, analysis of natural product diversity with respect to ecosystem level is worthwhile (Pietra, 2002). Other aspects such as unculturable species, life under extreme conditions, and management including threat management of natural product diversity are also discussed by this author.

On the basis of their extraordinary species diversity, which is reflected by the fact that insects represent more than 50% of all described organisms, whole insects and other arthropods and the substances extracted from them have been used worldwide as medicinal resources by human cultures (Costa-Neto, 2005). Their properties for example range from immunological, analgetic, anti-bacterial, anti-coagulant, anti-cancer, diuretic, and anesthetic to anti-rheumatic agents (Ahn et al., 2000; 2006; Costa-Neto, 2005). As with the use of insects as human food, many cultures especially in Asia (e.g. Pemberton, 1999) or Africa (e.g. Hemp, 2001) use arthropods as drugs and highly appreciate their therapeutic potential. In contrast western societies with their common entomophobia only utilize various products of honey bees (e.g. Dettner, 2007), anthraquinone pigments from homopteran insects (e.g. Schweppe, 1993), or cantharidin-containing insect extracts (see Dettner, 2007; Hoppe, 1977). Worldwide but especially in Eastern Asia arthropods therefore appear to represent an unexplored and unexploited, nearly inexhaustible source of drugs for modern medicine (Pemberton, 1999). Alone in Korean traditional medicine 19 species/taxa of insects which are successfully used are registered (Pemberton, 1999).

Apart from these de novo synthesized molecules, insects may contain many additional natural compounds which are often sequestered sometimes even chemically modified by the insect, but produced by associated bacteria, fungi or these natural compounds are derived from plants (Opitz and Müller, 2009) and even animals (Dettner et al., 1997). These compounds are often characterized by chemically complicated structures and may represent therapeutically valuable compounds.

However, most of these molecules have only been isolated from Petri dishes in the laboratory when bacteria or fungi were isolated from the insect hosts. In most cases these compounds have not even been shown to be present within the insect hosts. Therefore, the biological significance of these natural compounds in symbiotic or parasitic systems where insects represent hosts is usually not known

(Dettner, 2007). Nevertheless, it is extremely useful to gather knowledge on these compounds which often represent leading or key structures for therapeutically valuable compounds. Recently aposematically colored herbivorous insects were even used as guides to identify tropical plants that contain active compounds against cancer cell lines and protozoan parasites (Helson et al., 2009). Whereas insects with warning colors were restricted to active plants, inactive plants contained only few insect species with warning colors (Helson et al., 2009).

Interesting structurally novel bioactive substances were recorded from insect pathogenic fungi of the genus *Cordyceps* (Isaka et al., 2005; see Part I, Chapter 5). These approximately 400 species are parasites of insects and/or fungi which are found in wet tropical regions. The parasites exhibit a high degree of host specificity and were isolated from Hemiptera, Hymenoptera (ants, wasps, bees), and especially lepidopteran larvae and pupae (Isaka et al., 2005; Paterson, 2008). Combinations of dead infested insects and fungal fruit bodies of *Cordyceps* are used as traditional Chinese medicine (Paterson, 2008). Other fungi from insects such as beetles, termites, ants, or wasps represent ecto- or endosymbionts which have to metabolize and to detoxify toxic compounds (Dowd, 1992). It is of interest that these fungi represent a promising source of detoxifying fungal enzymes (Dowd, 1992).

In addition insects contain a huge array of larger sized molecules which are biologically active. For example, it is vital for blood-sucking arthropods that vertebrate blood remains in a liquid form. Therefore blood suckers such as bugs, lice, sandflies, mosquitoes, or biting flies excrete anticoagulants with their saliva (Lehane, 2005) which may contain anti-coagulant proteins as in tabanid flies (Ahn et al., 2006). Various insect peptides may have biological activities which range from antimicrobic (Vilcinskas and Gross, 2005), to anti-viral and anti-tumor (Chernysh et al., 2002) activities (see Part I, Chapter 3, Part II, Chapter 7). Another strategy in the search for new biologically active molecules from insects is the biochemical study of species from extreme habitats. Very promising examples are larvae from the petrol fly genus *Helaeomyia* which live in naturally occurring petroleum pools. The fly larvae contain many interesting bacterial strains and enzymes which may for example tolerate various organic solvents (Kadavy et al., 2000, see also Dettner, 2003). Other enzymes derived from insects may even be pesticide-degrading. Another example is the medicinal maggot of *Lucilia sericata*, a larva which cleans chronic wounds not only mechanically but also chemically ("biosurgery"; Fleischmann et al., 2004; see Part I, Chapter 4). They externalize a lot of active low molecular compounds and show genes that are differentially expressed in response to septic wounds and may also be responsible for the production of anti-microbial peptides (Altincicek and Vilcinskas, 2009). It was also shown that saprophagous and coprophagous maggots of the drone fly *Eristalis tenax* can survive extreme microbial stress through induction of new genes (e.g. for anti-microbial peptides) in response to septic injury (Altincicek and Vilcinskas, 2007).

Low-molecular compounds produced by insects that are biologically active and have potential practical applications are initially presented below. Various synthetic analogues of the toxin cantharidin are also presented, illustrating that the therapeutic potential of such compounds is tremendous, should the toxicity of the original

compound be too high. Subsequently presented are mostly low molecular weight metabolites from microorganisms which were produced in the laboratory and previously isolated from certain host insects. Usually the compounds compiled are also characterized by their formula, molecular mass, and LD_{50}-values as far as these data were available. Some of these compounds are also produced by microorganisms from non-insect sources.

6.2 Interesting Low Molecular Natural Compounds from Insects and Their Biologically Active Synthetic Derivatives

6.2.1 Cantharidin from Coleoptera and Canthariphilous Insects and Its Natural and Synthetic Analogues

The unusual terpenoid compounds cantharidin, palasonin and their natural analogues have been identified both in animals and plants. Cantharidin, a monoterpene anhydride (2-endo, 3-endo-dimethyl-7-oxabicyclo[2.2.1]heptane-2-exo,3-exo-dicarboxylic anhydride; $C_{10}H_{12}O_4$; MM: 196.20; LD_{50}: 1.0 mg/kg mice intraperitoneal; Fig. 6.1) occurs in the phylogenetically related meloid (Meloidae) and oedemerid (Oedemeridae) beetles and serves as a defensive respectively toxic compound. In other insects including the so-called canthariphilous insects but also in cantharidin-producing meloids (Nikbakhtzadeh et al., 2007) the toxic and volatile cantharidin is detected, ingested, detoxified, sequestered and often transferred into eggs, larvae, and pupae of following generations (Dettner, 1997; Hemp et al., 1999; Eisner et al., 2005). Palasonin ($C_9H_{10}O_4$; MM: 182.17; LD_{50}: 7.5 mg/kg mice subcutaneous; Fig. 6.1) or demethyl-cantharidin, which represents a chiral molecule as compared with cantharidin, was recorded from flowers, fruits, and seeds of *Butea*

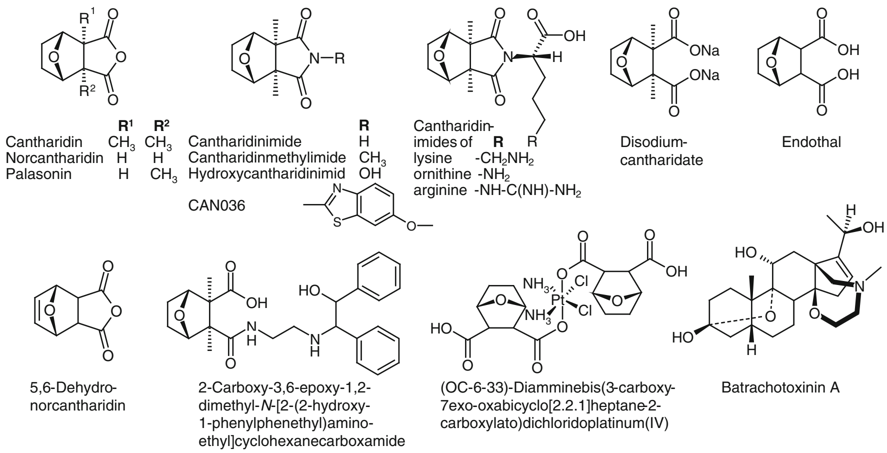


Fig. 6.1 Natural and synthetic cantharidin analogues together with batrachotoxinin A

frondosa (Fabacea; = *Butea monosperma*), and extracts especially of the seeds were formerly used as anthelmintica and insecticides (Bochis and Fisher, 1968). Obviously cantharidin is biosynthetically derived from farnesol (McCormick and Carrel, 1987), whereas palasonin could be produced from cantharidin by oxidative demethylation (Fietz et al., 2002). Recently, palasonin together with cantharidin- and sometimes palasonin-imides (Fig. 6.1) have been recorded from cantharidin-producing insects such as meloid beetles (*Hycleus*: Dettner et al., 2003; Mebs et al., 2009; *Mylabris*: Nikbakhtzadeh and Ebramihi, 2007), but also from those insects which feed on cantharidin-containing food (Fietz et al., 2002). In the pods of *B. frondosa* palasimide (see Fig. 6.1) was also found (Guha et al., 1990). It is interesting that *B. frondosa* contains (*S*)-(–) palasonin (>99% ee) whereas insects produce palasonin of low ee with the (*R*)-(+)-enantiomer (0–50% ee) prevailing (Fietz et al., 2002). Additionally, also identified in *Mylabris* and partly in *Lytta* were cantharidinimide ($C_{10}H_{13}NO_3$; MM: 195.22) and three novel cantharidinimides, in which the anhydride oxygen atoms are replaced by the basic amino acid moieties of L-lysine ($C_{16}H_{24}N_2O_5$; MM: 324.37; Fig. 6.1), L-ornithine ($C_{15}H_{22}N_2O_5$; MM: 310.35; Fig. 6.1), and L-arginine ($C_{16}H_{24}N_4O_5$; MM: 352.39; Fig. 6.1) (Nakatini et al., 2004).

Activity of cantharidin and analogues against protein phosphatases and cantharidin poisoning. It is highly interesting that cantharidin and almost all other cantharidin analogues are biologically extremely active and represent typical leading structures for example for medicaments. The terpene anhydride cantharidin inhibits protein phosphatase 2a (Li and Casida, 1992) and shows phlogistic, vesicant, toxic, and aphrodisiac (only in certain insects, see Dettner, 1997) effects (Liu et al., 1995; Matsuzawa et al., 1987). By radioligand binding studies with mouse tissues and especially mouse liver cytosol Graziano et al., (1988) investigated the toxicity of cantharidin and a lot of analogues to mice. They found that toxicity of cantharidin and related oxabicycloheptanes including the herbicide endothal ($C_8H_{10}O_5$; MM: 186.16; LD_{50}: 14 mg/kg mice intraperitoneal; Fig. 6.1) is attributable to binding at a specific site in the liver and possibly other tissues.

In humans clinical signs of cantharidin poisoning are nonspecific. Observations after ingestion include burning sensations of the lips, mouth and pharynx, blister formation followed by injury of the gastrointestinal tract and renal failure. Treatment of human cantharidin intoxication is largely supportive and there is no known antidote. After oral ingestion of cantharidin vomiting should be induced, and fatty materials such as milk should be avoided. There is no evidence that charcoal binds cantharidin. After topical exposures with cantharidin, the affected area should be cleaned with organic solvents or fatty soap in order to dilute the cantharidin (Moed et al., 2001).

Therapeutic activity of cantharidin and natural and synthetic analogues against vertebrate tumor cells. Cantharidin inhibits various tumor cell lines (e.g. HeLa cells, murine ascites hepatoma, reticulocell sarcoma) and it was often applied in humans especially those suffering from primary hepatoma. Moreover, as compared with many other natural compounds cantharidin was shown to represent a novel and potent multi-drug resistance reversal agent and therefore may be a potential adjunctive agent for tumor therapy (Zheng et al., 2008). However, its severe cytotoxicity

towards mucous membranes, the gastrointestinal tract, ureter, and kidney prevented further application (Shan et al., 2006). Therefore, a lot of analogues of cantharidin were synthesized in the hope that they were therapeutically highly effective whilst showing only moderate to low toxicities. It has been recently shown that internally applied cantharidin showed reduced systemic toxicity if it was encapsulated into pegylated liposomes. This treatment led to a decrease in the toxicity without affecting anti-tumor activity against human breast cancer MCF-7 cells (Chang et al., 2008).

Extracts from these beetles which contain cantharidin possess anti-tumor properties, increase the number of leucocytes, and have irritant effects on the urinary organs. In the search for less toxic analogues of cantharidin, disodium cantharidate was first identified ($C_{10}H_{12}O_5Na_2$; MM: 258.18; LD_{50}: 3.4 mg/kg mice; orally; Fig. 6.1), along with its demethylated form which is called norcantharidin ($C_8H_8O_4$; MM: 168.15; LD_{50}: 4–12.5 mg/kg mice intraperitoneal; Fig. 6.1). At concentrations of 1 μl/ml, disodium cantharidate inhibited growth of HeLa-, CaEs-17 (human esophageal carcinoma)- and BEL-7402 (human hepatomal)-cells and was used in several clinical studies in China (Wang, 1989). Norcantharidin has been used in cancer therapy in China since 1984. It showed the least nephrotoxic and inflammatory side-effects (Liu et al., 1995) and inhibited the proliferation of various tumor cell lines (HeLa, CHO, CaEs-17, BEL 7402, SMMC7721 human hepatoma, HEP-2, human epidermoid laryngocarcinoma) and transplanted tumors (embryonal adenocarcinoma, hepatoma; see Liu et al., 1995). In addition norcantharidin may stimulate hemopoiesis and immunity directly and via cytokines and suppresses the growth of human leukemic cells (Liu et al., 1995). Its effects on cytokinetics, the cytoskeletal system, and cell division were studied in detail (Wang, 1989). Also 5,6-dehydronorcantharidin (Fig. 6.1) was recognized as a potential anti-cancer agent (especially against EAC cells; Wang, 1989) and both hydroxycantharidinimide ($C_{10}H_{13}NO_4$; MM: 195.22; Fig. 6.1) and cantharidin-methylimide ($C_{11}H_{15}NO_3$; MM: 209.24; Fig. 6.1) represent less toxic cantharidin analogues, effective against some primary hepatomas and with anti-tumor activities against the KB cell line (Wang, 1989). It is also notable that introducing a 2-aminobenzothiazole-group into the 6-position of cantharidin resulted in synthetical analogues such as CAN036 (Fig. 6.1) which enhance the cytotoxicity to cancer cells while reducing the non-malignant cell toxicity (Kok et al., 2007). Compared with endothal two synthetic endothal-platinum (IV) complexes including compound (OC-6-33)-diamminebis(3-carboxy-7exo-oxabicyclo[2.2.1]heptane-2-carboxylato)dichloridoplatinum(IV) (Fig. 6.1) show a higher cytotoxicity against human cancer cell lines of ovarian carcinoma (CH1), cervical carcinoma (HeLa), and osteosarcoma U-2 OS as compared to endothal (Reithofer et al., 2008).

Finally, it was found that the two methyl groups of cantharidin are not the main functional groups responsible for anti-tumor activity and for the stimulation of bone marrow but that these methyl groups are associated with urinary irritation. Further compounds such as hydroxycantharidinimide (Fig. 6.1), cantharidin-methylimide (Fig. 6.1), and dehydronorcantharidin ($C_8H_6O_4$; MM: 166.83; LD_{50}: 14 mg/kg mice intraperitoneal; Fig. 6.1) showed high biological activities but low toxicities.

Therapeutic activity of cantharidin in treating human cardiac failure. As indicated by Moed et al. (2001) cantharidin acts as a vasoconstrictor and positive inotrope in guinea pigs and human cardiac tissue in vitro. It was suggested that safer synthetic cantharidin derivatives may be used in the future for the treatment of human cardiac failure.

Activity of cantharidin on vertebrate skin. It is interesting to note that cantharidin, which had been removed from the market for a long period of time, is today used again in dermatology in order to treat warts and molluscum (Moed et al., 2001). In former times the compound was topically and internally used in both human and veterinary medicine (Vogel, 1886). Very promising results were recently reported for treatment of molluscum contagiosum which is a viral infection of the skin or occasionally of the mucous membranes. Cantharidin treatment was obviously superior as compared with many other potential treatments such as the immune modulator imiquimod, cryotherapy, or curettage (Coloe et al., 2009). In spite of the fact that more than 90% of patients were satisfied with cantharidin's efficacy nearly 80% reported side-effects with discomfort, pain, and blistering (Coloe and Morrell, 2009). It must also be remarked that cantharidin (1%) in admixture with podophyllotoxin (5%) and salicylic acid (30%) was safe and effective in the topical treatment of simple and mosaic plantar warts respectively papilloma which represent benign epithelial tumors (Bengoa Vallejo et al., 2008). Even in facial flat warts topical treatment with 0.7% cantharidin solution was safe and effective and all patients were clinically cured within 16 weeks (Durmazlar et al., 2009). When cantharidin is absorbed by epidermic cells, serine proteases are activated and released, which results in degeneration of desmosomal plaque. This leads to acantholysis, intraepidermal blistering, lysis of the skin (Moed et al., 2001), and dermal perivascular leukocyte infiltration (Morris et al., 2009). In the United States there are purchasable cantharidin solutions such as cantharone® or cantharone® plus. These medications form a blister under the wart thus killing the wart by cutting off its blood supply.

The synthetic cantharidin analogue 2-carboxy-3,6-epoxy-1,2-dimethyl-*N*-[2-(2-hydroxy-1-phenylphenethyl)aminoethyl]cyclohexanecarboxamide ($C_{26}H_{32}O_5N_2$; MM: 452.54; Fig. 6.1) exhibited a topical anti-inflammatory activity which was recorded as the ability to inhibit cantharidin-induced inflammation in immature rats (Fryer et al., 1977).

Cantharidin and blistering. Cantharidin blisters (which are formed 24–48 h after topical cantharidin treatment) and analysis of their interstitial fluids was used to measure the amount of orally or intra-venously administered drugs such as antibiotics that may effectively reach the interstitial fluids (Fortenbach et al., 2008). Additionally, effects of low-dose aspirin on acute inflammatory responses by applying cantharidin externally, were measured in humans (Morris et al., 2009). Small blisters may be induced by external application of blistering solutions such as cantharone® or cantharone® plus. Certainly treatment of mucous membranes is contraindicated and application near eyes and eyelids should be avoided.

Activity of cantharidin on plants. Cantharidin has been reported to influence plant growth in various ways: Aqueous solutions inhibited growth of various plant seedlings, inhibited root development, acted as selective mitotic inhibitors, and induced necrotic lesions and collapse of leaves (Cutler, 1975). Recent data indicate

that treatment of wild-type *Arabidopsis* seedlings with protein kinase inhibitors such as cantharidin delay the asymmetric expression of the green fluorescent protein at the root tip after gravistimulation (Sukumar et al., 2009). In addition cantharidin also acts on mosses such as *Hypnum plumaeforme*. Concentrations of momilactone A und B, which play an important role in defense responses against biotic and abiotic stress conditions were increased by treatment with both cantharidin and jasmonic acid (Kato-Noguchi and Kobayashi, 2009).

Activity of cantharidin on Fungi. Type 2A Ser/Thr phosphatases (PP2As) are involved in the regulation of the variety of cellular process in *Sclerotinia sclerotiorum*, a necrotrophic, omnivorous plant pathogen with worldwide distribution. It was recently shown that in the presence of cantharidin hyphal elongation and numbers of sclerotia were impaired whereas sclerotial size increased (Erental et al., 2007).

Activity of cantharidin against viruses. Romero et al. (2007) tested the effect of cantharidin and other compounds on "in vitro" models of hepatitis B virus (HBV) and bovine viral diarrhea virus (BVDV) replication. It was shown that cantharidin induced toxicity in EBTr cells and had no protective effect against BVDV. In contrast the anhydride was able to inhibit HBV production at concentrations 10- to 100-fold lower than that inducing cell toxicity which suggests that cantharidin is useless for the treatment of infection with flaviviruses, but potentially useful in combined therapy against hepatitis B.

6.2.2 Other Insect-Derived Compounds

Batrachotoxins were recently found in certain passerine birds of New Guinea and it was suggested that the birds sequester these neurotoxic steroidal alkaloids from dietary sources (Dumbacher et al., 2004). High levels of several batrachotoxins were recorded in representatives of the genus *Choresine* which belongs to the Melyridae (Malachiidae) beetle family. Batrachotoxinin A ($C_{24}H_{35}NO_5$; MM: 417.55; LD_{50}: 1 mg/kg mice subcutaneous; Fig. 6.1) is not as toxic compared with the esters such as batrachotoxin (LD_{50}: 0.2 μg/kg subcutaneous) which is nearly as toxic as strychnine and acts on the voltage-dependent sodium channel of nerve and muscle. It binds to an open form of the sodium channel and prevents closing of the channel. Various data exist on the biological activity of batrachotoxins (Daly et al., 1999). Many applications concern the effects of drugs on voltage-dependent sodium channels (Daly et al., 1999). At the moment one must suppose that both neotropical dendrobatid frogs of the genus *Phyllobates* and Guinean birds receive their batrachotoxins via the food chain from the malachiid beetles. On the other hand it is also possible that the beetles either contain symbionts or must feed on steroids since the steroidal skeleton cannot be built up by insects in general (Dettner, 2007; Dumbacher et al., 2004).

Insects and other arthropods contain a huge number of de novo produced low molecular compounds which are used as defensive compounds and toxins (Eisner et al., 2005; Blum, 1981; Dettner, 2010; Francke and Dettner, 2005; Laurent et al., 2005). Apart from the terpene cantharidin (Section 1.2) these often well known and chemically simple constituents usually are of low therapeutic value because they

often are too toxic for humans and other targets. A considerable number of de novo produced larger natural compounds also exist which may be of higher therapeutic value.

Many other chemically more complicated insect defensive compounds and hemolymph toxins exist (Blum, 1981; Eisner et al., 2005; Francke and Dettner, 2005; Laurent et al., 2005). Usually these are produced by the insects (Morgan, 2004), however it is unknown if microorganisms might be involved in the biosynthesis of these natural products.

6.3 Low Molecular Weight Compounds from Insect-Derived Microorganisms

Many insects harbor microorganisms which range from ubiquitous gut bacteria to symbiotic species which are associated with gut, body cavity and even mycetocytes and mycetomes (Baumann et al., 2006; Dettner and Peters, 2003). Starting with the pioneering work of Paul Buchner (Buchner, 1953) many recent publications exist dealing with systematics, genomics, and evolution of insect symbionts (e.g. Bourtzis and Miller, 2003, 2006, 2009; Moran et al., 2008) or their molecular interactions with their hosts (Dale and Moran, 2006).

Symbiotic microorganisms usually cannot be cultivated apart from selected facultative symbionts (Pontes and Dale, 2006). Therefore, in comparison with other potential drug-candidates a continuous supply of many natural compounds for clinical or greenhouse tests ranges from very difficult to impossible.

Taxonomically there is a large diversity of microorganisms which are detected or isolated from insects. Since taxa such as the *Actinomycetes* species are able to produce a lot of highly biologically active molecules (Grabley and Thiericke, 2000) one main task in the future should be to isolate and characterize new and "exotic" *Actinomycetes* species from insects or arthropods. Their metabolic capabilities should be intensively studied under various side conditions such as oxygen contents, pH-values or varying nutrition supplies.

In the following low molecular weight compounds from insect-derived microorganisms are compiled according to their host groups among insects. Only pyocin (Section 6.3.3) and an insecticidal protein from Neuroptera (Section 6.3.5) are characterized by higher molecular weights. It is evident that most data are based on cultivable microorganisms which were previously isolated from open insect compartments such as gut or accessory glands. Various aspects of intestinal microorganisms in invertebrates and especially insects including methodical works were thoroughly presented by König and Varma (2006). Additionally, observations on metabolic activities of true ekto- and endosymbionts and their hosts also exist (Piel, 2004, 2009).

Section 6.3 deals with systems consisting of host insect and smaller microorganisms. However, there also exist systems including three species. Entomopathogenic nematodes of genera *Heterorhabditis* and *Steinernema* can kill their host insects within two days of infection. The infective juvenile soil-dwelling stage is associated

with symbiotic gut bacteria of genera *Xenorhabdus* and *Photorhabdus* that are pathogenic to insects, killing them by septicemia. These insecticidal proteins, hydrolytic enzymes, and various highly interesting and biologically active secondary metabolites were compiled in detail by Bode (Chapter 5, this volume).

6.3.1 Odonata (Dragonflies)

Amicoumacin B ($C_{20}H_{28}O_8N_2$; MM: 424.45; LD_{50}: 132 mg/kg mouse, perorally; Fig. 6.2): This pseudopeptide antibiotic is based on 8-hydroxyisocoumarin. It was isolated from a *Bacillus* species which was found in the gut of a *Coenagrion* dragonfly larva (Gebhardt et al., 2002). The compound shows anti-inflammatory (rat), anti-ulcer (man: stomac), and herbicidal (*Lemna*) activities and is used as an acaricide (Itoh et al., 1982). Furthermore, the carboxylic amide targets bone morphogenetic protein 2, which plays an important role in the development of bone and cartilage (Sun et al., 2009). In addition, amicoumacin B has potent gastroprotective activity when administered intraperitoneally. Three amicoumacins A–C (Itoh et al., 1982) together with their 8'-phosphate ester derivatives (Hashimoto et al., 2007) are produced by *Bacillus pumilus*; amicoumacin B is also produced by *Nocardia jinanensis* (Sun et al., 2009).

6.3.2 Orthoptera

Hirsutellic acid A ($C_{29}H_{40}N_4O_5$; MM: 524.65). From the entomopathogenic fungus *Hirsutella* species which was collected from cricket leaf litter the linear tetrapeptide

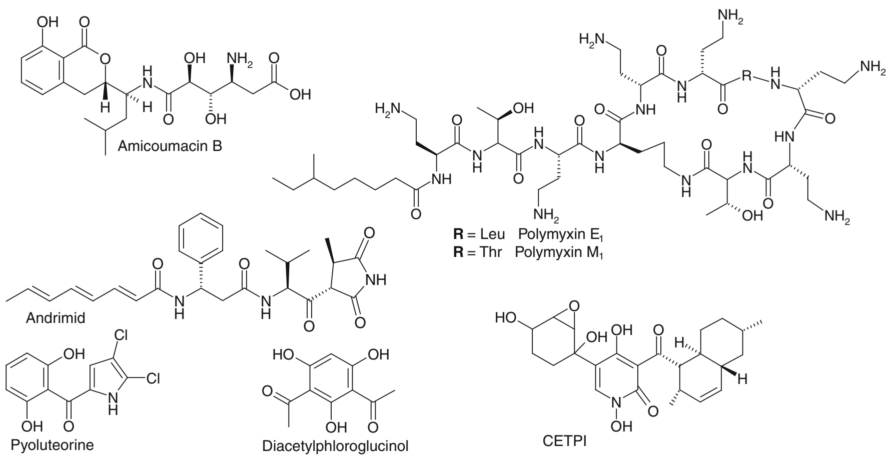


Fig. 6.2 Secondary compounds from bacteria and fungi isolated from Odonata, Orthoptera, Hemiptera, and Hymenoptera

hirsutellic acid A was isolated (Thongtan et al., 2006; Chapter 5 by Bode, this volume). The compound is characterized by an anthranilic acid residue at the C-terminus and exhibits activity against the malarial parasite *Plasmodium falciparum* K1 with an IC_{50} value of 8.0 μM. However, it was nontoxic against African green monkey kidney fibroblasts (Vero) (Thongtan et al., 2006).

6.3.3 Hemiptera

Polymyxins E_1 (Fig. 6.2) and M_1 (Fig. 6.2) (P.E_1: $C_{53}H_{100}N_{16}O_{13}$; MM: 1.150; P.$M_1$; MM: 1.156). The cyclic decapeptide antibiotics polymyxin E_1 (also called colistin A) and polymyxin M_1 (also called mattacin or polymyxin A_1) act exclusively against Gram-negative bacteria (Ullmann, 2003). The detergent-like, highly positively charged molecules damage membranes of growing and non-growing cells of Gram-negative bacteria by binding to lipopolysaccharide and phospholipids of the membranes. Both compounds differ by threonine (P. M_1) respectively leucine (P. E_1) in the seven amino acid ring. From the various described polymyxin molecules only two are medically important (polymyxin B, E). Polymyxin E_1 was isolated from *Bacillus* species and *B. polymyxa* derived from the brown winged green bug *Plautia stali* (Pentatomidae; Kenny et al., 1989; Kirk and Othmer, 1993). Polymyxin M_1 could be found in a *Bacillus* species isolated from rice brown planthopper *Nilaparvata lugens* (Delphacidae; Jigami et al., 1986).

Andrimid ($C_{27}H_{33}N_3O_5$; MM: 479.57; Fig. 6.2): The non-ribosomal peptide-polyketide antibiotic was isolated from culture broth of an intra-cellular bacterial symbiont associated with the rice brown planthopper *N. lugens* (Delphacidae; Fredenhagen et al., 1987). Interestingly the antibiotic activity of andrimid is highly specific against rice pathogens (e.g. *Xanthomonas*) but inactive or weakly active against various Gram-positive and negative bacteria. It was suggested that intracellular symbionts might produce anti-microbial substances in those insects lacking immunological defensive mechanisms. Other studies revealed an anti-bacterial activity against both Gram-positive and Gram-negative bacteria, however andrimid exhibited no anti-proliferative activity and a moderate activity was only detected against ovarian tumor cell lines (Singh et al., 1997). Remarkably andrimid also was found in both total extracts of marine sponges and bacterial isolates of the genus *Vibrio* which were isolated from the sponge *Hyatella* species (Oclarit, 1997). Additionally, also described were anti-bacterial derivatives (pseudopeptide pyrrolidine dione antibiotics) of andrimid and chemically similar natural compounds such as moiramides A–C, which were isolated from *Pseudomonas fluorescens* from a marine tunicate (Needham et al., 1994). Further sources for andrimid and moiramide B were *Enterobacter* sp. and *P. fluorescens* (Freiberg, 2005). Also in *Enterobacter cloacae* an *anrF* biosynthetic gene cluster was identified which is responsible for the production of andrimid (Yu et al., 2005). In addition the molecular target of these novel broad-spectrum antibacterials was identified as acetyl-CoA carboxylase, which is involved in inhibiting the fatty acid biosynthesis in bacteria (Freiberg et al., 2004) with sub-micromolar potency (Liu et al., 2008).

Pyoluteorin ($C_{11}H_7Cl_2NO_3$; MM: 272.08; Fig. 6.2). This pyrrol-antibiotic was isolated from unknown intra-cellular bacteria from the White-backed planthopper *Sogatella furcifera* (Delphacidae). Usually this polyketide is produced by *P. fluorescens* and *P. aeruginosa* and exhibits anti-bacterial, anti-fungal and herbicidal activities (Kenny et al., 1989; Steglich et al., 1997).

Pyocin R (MM: 1×10^7). Pyocines represent post-translationally modified bactericines respectively lantibiotics of *Pseudomonas* species which are heavy (R-type with MM: of 10^7) or light weight (S-type with MM: of 10^5; Bergan, 1975). They resemble phage tails and were studied by electron microscopy. R-type pyocin was produced by *P. fluorescens* which was isolated from rice brown planthopper *N. lugens* (Delphacidae; Kenny et al., 1989). Like bacteriophages these pyocines exert their bactericidal activities after adsorption on the cell surface to specific receptors of sensitive bacteria. Obviously these compounds are important in microbial ecology because they may protect the producing bacteria from pathogens.

Diacetylphloroglucinol ($C_{10}H_{10}O_5$; MM: 210.18; Fig. 6.2), which also occurs in the scolytid beetle *Scolytoplatipus mikado* (see Section 6.3.6), was isolated from unknown intra-cellular bacterial symbionts of the White-blacked planthopper *S. furcifera* (Kenny et al., 1989). There was identified a gene cluster for synthesis of this antimicrobial, fungicidal and phytotoxic polyketide in *Pseudomonas fluorescens* (Bangera and Thomashow, 1999). Recently there was found a *hfq*-gene in *P. fluorescens* which is responsible for production of both polyketide 2,4-diacetylphloroglucinol and the quorum-sensing signalling molecule *N*-acyl-homoserine lactone (Wu et al., 2010).

6.3.4 Hymenoptera

Offspring of the European beewolf *Philanthus triangulum* are protected from fungal infection by pathogens through symbiotic bacteria of *Streptomyces philanthi* (Kaltenpoth et al., 2006). The bacteria are located in the antennal glands of female wasps belonging to several *Philanthus* species. They are applied to the brood cells prior to oviposition and obviously produce antibiotics (Kaltenpoth et al., 2005).

CETPI ($C_{24}H_{32}O_7N$, MM: 446; Fig. 6.2). From a *Cytospora* fungus associated with *Pogonomyrmex badius* ants a cholesteryl ester transfer protein inhibitor was isolated (CETPI; Lee et al., 1996). These compounds may reduce the risk of coronary heart disease by raising HDL level and lowering LDL level at the same time. These CETP-inhibitors may represent a new approach for treating hypoalphalipoproteinemia patients who are at particularly high risk by having low levels of HDL-cholesterol. Therefore, several companies have developed potent inhibitor classes that reduce CETP activity and raise HDL-cholesterol after dosing in animal models (Sikorski, 2006).

Leaf-cutting ants live symbiotically with fungi of the genus *Leucoagaricus* which they grow with harvested leaf material. Subsequently the fungi represent the main food source for the ants. In order to protect their fungal gardens from pathogenic fungi such as *Escovopsis* the ants produce anti-fungal compounds via symbiotic

bacteria. From various microorganisms which are associated with leaf-cutting ants the macrolide candicidin D was identified which is highly active against *Escovopsis* but does not significantly affect the growth of the symbiotic fungus (Haeder et al., 2009). It has also been shown that the candicidin biosynthesis gene cluster is widely distributed among *Streptomyces* spp. (Jørgensen et al., 2009). Moreover, in *Pseudonocardia* species which are associated with the ant *Apterostigma dentigerum* the production of the cyclic depsipeptide dentigerumycin was observed (Oh et al., 2009a). This compound has highly modified amino acids and selectively inhibits the associated parasitic fungus *Escovopsis* species. From a *Burkholderia* species which was isolated from colonies of *Atta sexdens* a potent anti-fungal agent was isolated that inhibited entomopathogenic fungi, however mutualists were unaffected (Santos et al., 2004). It seems that the associations between attine ants and their actinomycete symbionts are less specific than previously thought, because a high percentage of isolated strains inhibited the growth of the parasitic fungus *Escovopsis* (Kost et al., 2007).

6.3.5 Neuroptera

The saliva of *Myrmeleon* larvae (Myrmeleontidae) contains insecticidal proteins which may paralyze prey (Yoshida et al., 2001). It was shown that these insecticides in reality are produced by the endosymbiont *Enterobacter aerogenes*. The protein had a molecular mass of about 63 KDa, and partial amino-acid sequencing indicated that it was the chaperonin GroEL homologue (Yoshida et al., 2001). In the meantime the microbiota of *Myrmeleon mobilis* with its closed midgut were characterized (Dunn and Stabb, 2005). In addition from *Bacillus cereus* which was isolated from *Myrmeleon* larvae a 34-kDa-insecticidal sphingomyelinase C was isolated and characterized which can paralyze German cockroaches (Nishiwaki et al., 2004).

6.3.6 Coleoptera (Beetles)

Maculosin ($C_{14}H_{16}N_2O_3$; MM: 260.29; Fig. 6.3): This phytotoxic and cytotoxic diketopiperazine = cyclo(-L-Pro-L-Tyr) and was isolated from *B. pumilus* from the foregut of the water beetle *Laccophilus minutus* (Gebhardt et al., 2002). Maculosin or more exactly Maculosin I was also isolated from various other microorganisms, the fungus *Alternaria alternata* and marine sponges. Maculosin is a host-specific phytotoxin from the *Alternaria*-weed pathogen causing black leaf blight in *Centaurea maculosa* (Stierle et al., 1988). Also Maculosin II (the dehydroxylated maculosin I) and various synthetic analogues may inhibit the growth of wheat coleoptiles (Bobylev et al., 2000).

Pederin derivatives, i.e. pederin ($C_{25}H_{45}NO_9$; MM: 503.63; LD_{50}: 0.14 mg/kg rat intraperitoneal; Fig. 6.3), pseudopederin ($C_{24}H_{43}NO_9$; MM: 489.59; Fig. 6.3), and pederone ($C_{25}H_{43}NO_9$; MM: 501.61; Fig. 6.3) are found in the hemolymph

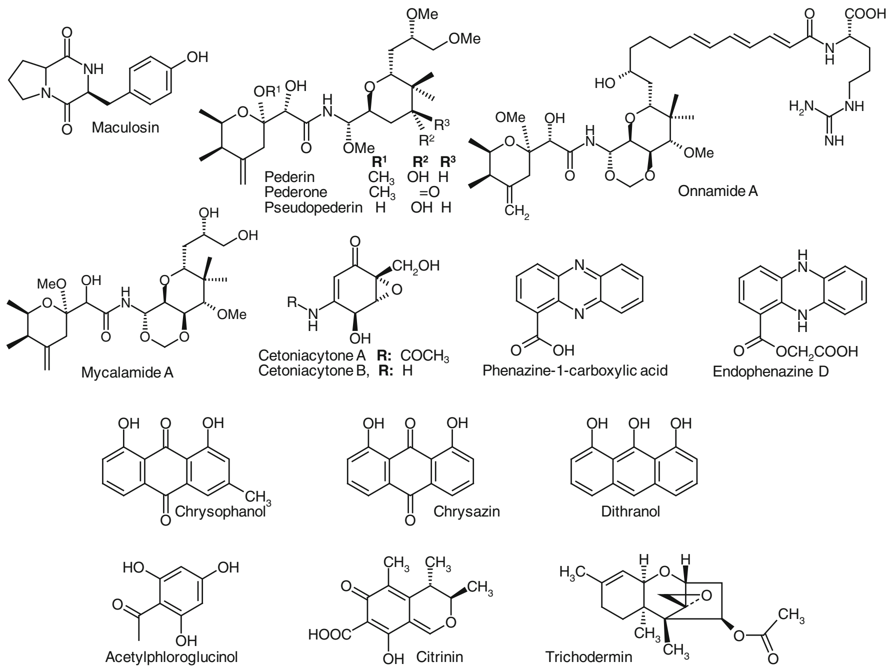


Fig. 6.3 Secondary compounds from bacteria and fungi isolated from Coleoptera respectively marine fungi (onnamide, mycalamide)

of rove beetles of the genus *Paederus* (Staphylinidae). These chemicals may effectively protect adults, larvae, pupae, and eggs from predation by many spider species (Kellner and Dettner, 1995, 1996). Furthermore, pederin causes severe inflammation and blistering on human skin, which is known as *Paederus*-dermatitis (in the eye: Nairobi eye). The toxicity of pederin is related to its inhibition of protein biosynthesis (prevents translation of mRNA at the ribosome) and cell division. It was reported that elderly patients with chronic necrotic and purulent sores completely recovered after treatment with minute amounts of pederin. Its use as an anti-cancer agent has been suggested based on the ability to block mitosis in normal and tumor cells at doses of 1 ng/ml and on reports that it inhibited sarcoma-180 tumors in mice (Narquizian and Kocienski, 2000). Like pederin its analogues mycalamides (Fig. 6.3), onnamides (onnamide A: $C_{39}H_{63}N_5O_{12}$; MM: 793.94; Fig. 6.3), theopederines, and icadamides which were isolated from marine sponges also may induce severe dermatitis. All of them are antiviral and exhibit anti-tumor activity. Finally, it was proved by investigating different morphs/chemotypes of *Paederus*, that unculturable bacteria from the genus *Pseudomonas* are responsible for the production of the polyketide pederin (Kellner and Dettner, 1995; Kellner, 2002). This represents the first case where a fitness-relevant metabolite from an insect is virtually produced by bacteria. Additionally, *Pseudomonas* strains are also responsible for the production of similar polyketides in sponges.

Mycalamides A ($C_{24}H_{41}NO_{10}$; MM: 503.58; Fig. 6.3) and B ($C_{25}H_{43}NO_{10}$; MM: 517.61) reveal potent in vitro cytotoxicity and in vivo anti-tumor efficacy against several leukemia and solid tumor systems as well as anti-viral activity. These analogues inhibit in vitro replication of murine lymphoma P388-cells and human promyelocytic (HL-30), colon (HT-29) and lung (A549) cells. Mycalamide A was also active against B 16 melanoma, Levis lung carcinoma, M5076 ovarian carcinoma, colon 26 carcinoma, and the human MX-1 (mammary), CX-1 (colon), LX-1 (lung), and Burkitt's lymphoma tumor xenografts. It was confirmed, that mycalamides like pederin are protein synthesis inhibitors. Mycalamide A also disrupts DNA metabolism but does not intercalate into DNA itself. It is suggested that anti-tumor activity of these compounds is a consequence of protein synthesis inhibition (Narquizian and Kocienski, 2000). Many derivatives of Mycalamides A and B have been prepared. In conclusion it was found that the *N*-acyl aminal bridge is the pharmacophore. Moreover, the homoallylic acetal encompassing C_4-C_6, which is responsible for the acid lability of the natural product as well as its vesicant effects, is not necessary for their anti-tumor or anti-viral activity. The C_6 acetal function contributes to the high activity of the natural products. Finally, the presence of the hydroxyl group at C_7 with (*S*)-configuration is important for the high activity. The configuration of the aminal center is also important with the (*S*)-configuration at C_{10} being significantly more active as an anti-tumor agent than the (*R)*-epimer. However, compounds with the (*R*)-configuration remain potential anti-viral agents. The complex triocadecalin ring system characteristic of the mycalamides, onnamides, and theopederins is not essential for high activity since pederin with its simple monocyclic right half is one of the most active of the natural products. Additionally, the side chain at C_{15} tolerates considerable variation with little impact on activity (Narquizian and Kocienski, 2000). Recently it was shown that a symbiosis island is involved in the horizontal acquisition of pederin biosynthetic capabilities by the bacterial symbiont of *Paederus* beetles (Piel, 2002; Piel et al., 2004).

Cetoniacytone A and B (Cetoniacytone A = $C_9H_{11}NO_5$; MM: 213.18; Fig. 6.3; Cetoniacytone B = $C_7H_9NO_4$; MM: 167.12; Fig. 6.3) and related metabolites were isolated from an *Actinomyces* species from the hind gut of rose chafer *Cetonia aurata*. Both constituents showed significant growth inhibition against hepatocellular carcinoma (HEP G2 = GI_{50} = 3.2 µmol/l) and breast adenocarcinoma (MCF 7: GI_{50} = 4.4 µmol/l) (Schlörke et al., 2002). Cetoniacytone A possesses an unusual C_7-N-aminocyclitol moiety in its structure, in which the acetylated amino group is located at the C_2 position. Comparable core structures are found in the anti-rheumatoid arthritis agents, the epoxyquinomicins, which were isolated from *Amycolatopsis* cultures (Mahmud et al., 2007). A gene cluster responsible for the biosynthesis of Cetoniacytone A was isolated from the above-mentioned *Actinomyces* strain (Wu et al., 2009). The nucleotide sequence analysis of the 46-kb DNA-region revealed the presence of 31 complete ORF's (open reading frames), including genes predicted to uncode a 2-epi-5-epi-valiolon synthase (CetA), a glyoxalase/bleomycin resistance protein (CetP), an acyl transferase (CetD), an FAD-dependent dehydrogenase (CetF2), two oxidoreductases (CetF1,

CetG), two aminotransferases (CetH, CetM), and a pyranose oxidase (Wu et al., 2009).

Endophenazine D ($C_{15}H_{12}N_2O_4$; MM: 284.27; Fig. 6.3) and phenazine-1-carboxylic acid (Fig. 6.3 = tubermycin B, $C_{13}H_8N_2O_2$; MM: 224.22) were produced by *Streptomyces anulatus* which was isolated from the gut of the chrysomelid beetle *Exosoma lusitanica* (Gebhardt et al., 2002). Both compounds showed no anti-microbial activity however they inhibited selectively *Botrytis cinerea*. In addition both phenazines showed herbicidal properties against *Lemna minor*. Finally, it was shown that production of various phenazines by *S. anulatus* was highly dependent on fermentation (especially degree of aeration) conditions (Gebhardt et al., 2002).

Anthraquinones are found in scale insects (Coccoidea) and chrysophanol ($C_{15}H_{10}O_4$; MM: 254.24; Fig. 6.3), chrysazin ($C_{14}H_8O_4$; MM: 240.21; Fig. 6.3), and dithranol ($C_{14}H_{10}O_3$; MM: 226.23; Fig. 6.3) are present within all developmental stages of leaf-beetles (Chrysomelodae). Within the sub-family Galerucinae they are widely distributed and serve as deterrents against predators such as ants or birds (Hilker et al., 1992). By treating *Galeruca* beetles with broad spectrum antimicrobials it was shown that anthraquinone biosynthesis was not reduced and anthraquinones and anthrones were obviously produced by beetle enzymes. Therefore, these compounds were transferred via the eggs from one generation to the next (Pankewitz et al., 2007a). In addition within the eggs of the beetles apart from *Wolbachia* species no further 16S rDNA from bacteria was isolated which would be responsible for anthraquinone synthesis (Pankewitz et al., 2007b). It was fascinating to identify that different polyketide folding modes converge to an identical molecular architecture such as the anthraquinone chrysophanol. In eucaryotes (fungi, higher plants, insects, such as *Galeruca*) chrysophanol is formed via a folding mode which differs from the biosynthesis in actinomycetes (Bringmann et al., 2006).

Acetylphloroglucinol ($C_8H_8O_4$; MM: 168.15; Fig. 6.3) and Diacetylphloroglucinol ($C_{10}H_{10}O_5$; MM: 210.18; Fig. 6.2). 2,4-Diacetylphloroglucinol represents a polyketide metabolite which is usually produced by fluorescent *Pseudomonas* strains, where also the genetic basis of its production has been studied (see Section 6.3.3). The phenol derivative is phytotoxic, antimicrobic, and shows biocontrol activity against soilborne fungal plant pathogens. Both compounds could be isolated from an unknown ectosymbiotic fungus of the scolytid beetle *S. mikado* (Kenny et al., 1989), whilst the diacetyl derivative was also isolated from unknown intra-cellular bacterial symbionts of the White-blacked planthopper *S. furcifera* (Kenny et al., 1989).

From the southern pine beetle *Dendroctonus frontalis* there were isolated symbiotic *Streptomyces* species which produce the polyene peroxide mycangimycin (Scott et al., 2008; Chapter 5 by Bode, this volume). This compound selectively inhibits fungal antagonists (e.g. *Ophiostoma*), while mutualistic fungi such as *Entomocorticium* seem relatively resistant (Oh et al., 2009b).

Citrinin ($C_{13}H_{14}O_5$; MM: 250.25; LD_{50}: 105–112 mg/kg oral mice; Fig. 6.3). Citrinin was isolated from an unknown ectosymbiotic fungus of scolytid beetle

Indocryphalus pubipennis (Scolytinae: Xyloterini) (Kenny et al., 1989). Normally this antibiotic pentaketide is produced by many *Penicillium*- and *Aspergillus* species. On rice, cereals, and even within bread these fungi cause mycotoxicosis due to citrinin production. The nephrotoxin shows various phytotoxic, teratogenic, mutagenic, and cancerogenic activities (Fugmann et al., 1996–1999).

Trichodermin ($C_{17}H_{24}O_4$; MM: 292.36; LD_{50}: >1000 mg/kg oral mice; Fig. 6.3). The 12,13-epoxytrichothecene trichodermin (4*ß*-acetoxy-12,13-epoxy-9-trichothecene) was produced by an unknown ectosymbiotic fungus isolated from the ambrosia beetle *Platypus calanus* (Platypodidae; Kenny et al., 1989). The same compound is known from the fungi *Trichothecium sporulosum* and *T. roseum*, where it is produced via the sesquiterpene trichodiene (Cane, 1999). The compound represents a very potent inhibitor of protein synthesis in mammalian cells which is achieved by blocking the activity of peptidyl transferase required for termination (Wei et al., 1974). Dependent on toxin and animal species most of the more than 100 trichothecenes exhibit pronounced phytotoxic, insecticidal, anti-fungal, anti-viral and cytotoxic activities (Fugmann et al., 1996–1999).

6.3.7 Siphonaptera

Bacitracin A ($C_{66}H_{103}N_{17}O_{16}S_1$; MM: 1422.69; LD_{50}: 3750 mg/kg oral mice; Fig. 6.4). Bacitracin represents a cyclic peptide, the marketed mixture contains at least nine water-soluble peptides, some of which represent degradation products of bacitracin A (Kirk and Othmer, 1993). For example, in bacitracin B one of the isoleucines of bacitracin A is replaced by a valine. The bacterial strain was determined as a *Bacillus* species which was isolated from the tissue of a *Ceratophyllus* species (Gebhardt et al., 2002). Bacitracin is bactericidal for many Gram-positive

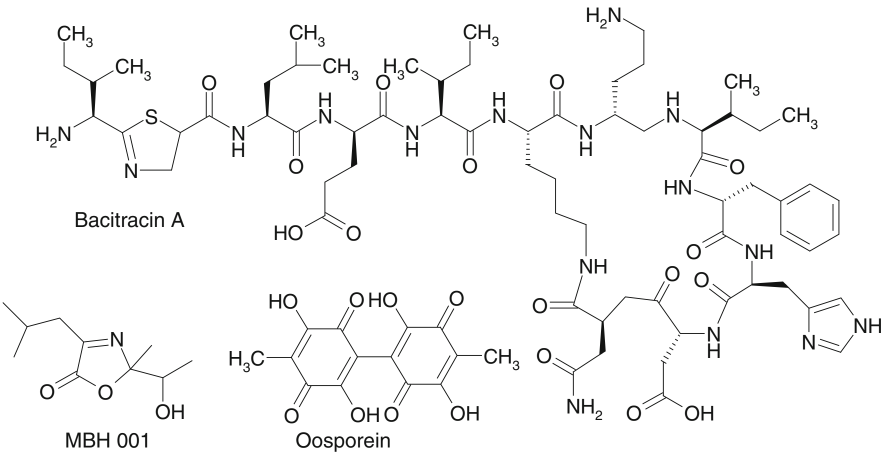


Fig. 6.4 Secondary compounds from bacteria and fungi isolated from Siphonaptera and unknown insects

rods and cocci and a few Gram-negative bacteria by blocking cell wall biosynthesis. In addition bacitracin showed a high anti-fungal activity and is nephrotoxic in humans, if it is applied topically. Today bacitracin is used as a topical bactericide in humans, especially in admixture with neomycin. Most of the worldwide material however is used as an animal food additive. An increasing number of *Staphylococcus aureus* strains are bacitracin-resistant (Kirk and Othmer, 1993).

6.3.8 Unknown Insects

MBH 001 ($C_{10}H_{17}O_3N$; MM: 199.25; Fig. 6.4). This metabolite which represents an herbicidal was isolated from an unknown microorganism cultivated from an unknown insect (Suguira et al., 1992). The substituted pentacyclic heterocycle exhibits excellent effects against diverse crops even in small doses.

Entomopathogenic fungi of *Beauveria bassiana* (Hypocreales) penetrate insect integuments and produce a large variety of polyketide and non-ribosomal peptide natural products that act as immunosuppressors and toxins (Xu et al., 2009). *B. bassiana* produces the cyclooligomer non-ribosomal depsipeptides beauvericin ($C_{45}H_{57}N_3O_9$; MM: 783.95) and bassianolide ($C_{48}H_{84}N_4O_{12}$; MM:L 909.2), the diketomorpholine bassiatin ($C_{15}H_{19}NO_3$; MM: 261.32), the cyclic peptides beauverolides (Chapter 5 by Bode, this volume), the dibenzoquinone oosporein (see below), and the 2-pyridone tenellin ($C_{21}H_{23}NO_5$; MM: 369.41) (see Chapter 5 by Bode, this volume).

Beauveria brongniartii (Ascomycota), another entomopathogenic fungus which may infest larvae of *Melolontha melolontha* (Common European Cockchafer), excretes a red toxic and anti-fungal metabolite called oosporein (Nagaoka et al., 2004). The dibenzoquinone pigment oosporein ($C_{14}H_{10}O_8$; MM: 306.23; LD_{50}: 6.12 mg/kg oral chick, see above; Fig. 6.4) could also be isolated from *B. bassiana* which originated from an infected whitefly (Eyal et al., 1994).

From fermentation broth of the entomopathogenic fungus *Metarhizium anisopliae* the polyketids aurovertin D and F–H were isolated (Aurovertin D: $C_{25}H_{32}O_9$: 476.52; Aurovertin F: $C_{23}H_{31}O_8$: 435.20; Aurovertin G: $C_{25}H_{32}O_{10}Na$: 515.18; Aurovertin H: $C_{25}H_{33}O_{10}$: 493.20; Azumi et al., 2008). In addition if the same fungus was grown on a medium with powdered silkworm pupae, the anti-bacterial compounds helvolic acid ($C_{33}H_{44}O_8$; MM: 568.68) and 1,2-dihydrohelvolic acid (Chapter 5 by Bode, this volume) could be detected. Moreover the entomopathogenic *M. flavoviride* produced two new diterpene pyrone-type compounds known as metarhizins A (see Chapter 5 by Bode, this volume) and B. Both compounds show potent and selective anti-proliferative activity against both insect and human cancer cell lines (see Chapter 5 by Bode, this volume).

From entomopathogenic bacteria of *Bacillus thuringiensis* subsp. *Kurstaki* and other *Bacillus* species was isolated the antibiotic zwittermycin A ($C_{13}H_{28}N_6O_8$; MM: 396.39) which represents a new class of antibiotics (Chapter 5 by Bode, this volume). In the former mentioned species, three zwittermycin A biosynthesis-related genes were detected (Kevany et al., 2009). The compound has the potential to suppress plant disease due to its broad spectrum activity against certain Gram-positive and Gram-negative eukaryotic microorganisms.

6.4 Conclusions

Because of their species diversity insects represent an exhaustless source of bacteria and fungi and their metabolites. In addition insect-derived natural compounds were found in huge numbers. If one considers chemically unusual and new metabolites from anuran skins (Daly et al., 1999; Dettner, 2007) which very often represent degradation products from their often unknown arthropod food (mites, ant, millipedes) it can be suggested that a lot of unknown arthropods especially in the tropics may contain unusual hitherto unknown natural products which must be elucidated and tested. It seems also of interest to extract metabolites and microorganisms from insects with unusual habitats and from taxonomically related and unrelated insects. In botany chemotaxonomically based pharmaceutical research has been well known for a long time, whereas comparative analyses of arthropods are relatively new. An important question on successful cultivation of microorganisms from insects in the laboratory is whether these compounds from the Petri dish are also present in the host insect. There is a need to stress cultivable microorganisms from insects in the laboratory in order to receive chemically different metabolites. Subsequently an analysis should be carried out of the conditions under which the microorganisms occur in the appropriate insect compartment.

Because of the predominantly unclear chemical nature of the relevant active compounds one has no idea whether these insect–microorganism interactions are parasitic or symbiotic. However, in the future it should be possible to analyze trace compounds of these systems in order to discover the key compounds as in *Paederus* beetles or in the fungus-growing Attini ants. Then it would be possible to manipulate and understand the system and to assess the biological and pharmaceutical activity of these natural compounds.

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Part II
Insect Biotechnology in Plant Protection

Chapter 7
Insect Antimicrobial Peptides as New Weapons Against Plant Pathogens

Carin Jansen and Karl-Heinz Kogel

Abstract One action to furnish the increasing demand for food and feed of the growing world population is to reduce yield losses caused by plant diseases. Biotechnology helps to generate crop plants with improved resistance against pathogenic bacteria, fungi, and pests. The expression of antimicrobial peptides (AMPs) from various sources is a promising approach to pursue strengthening plant health in a sustainable way. Antimicrobial peptides from insects have been shown to be effective also against bacterial and fungal plant pathogens. In the 1990s cecropin was the first AMP from insects that was ectopically expressed in various crops such as potato or rice to improve their disease resistance. Since then many efforts have been made to optimize AMPs from insects for their use in plant protection. For instance, sequence optimization to stabilize the peptides in plant cells or fusion of different peptides to combine their antimicrobial capabilities resulted in more efficient antimicrobial activity. Here, we summarize examples for strategies to use AMPs in plant biotechnology. Furthermore, we highlight some future tactics to use AMPs from insects to improve plant health in modern cropping systems on the basis of their significant effectiveness, their remarkable specificity, and their obvious economical potential.

Keywords Resistance · Plant defense · Antifungal · Innate immunity · Microbes

7.1 Controlling Microbial Plant Pathogens

Plant pathogens and pests cause severe and increasing crop losses worldwide amounting to $30–50 billion annually (Osuski et al., 2000; Cook, 2006). Among other strategies biotechnology approaches have been launched to support plant health, to stabilize yield, and to increase food safety with a considerable worldwide

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success (Christou et al., 2006). Peptides with activity against plant-infesting pests and infectious diseases have attractive potential for sustainable plant protection. One example already put into agricultural practice concerns the application of microbial peptides from diverse subtypes of the ubiquitous bacterium *Bacillus thuringiensis* (Bt) that show activity against various insects (Tabashnik et al., 2008). The global area of transgenic Bt crops that contain a Bt peptide has reached more than 25 million ha out of the 125 million ha of transgenic crops overall produced in 2008 (James, 2008). Recent strategies now focus on a combination of insecticidal Bt peptides with other traits like herbicide resistance thus enhancing the potential application of novel peptides. Though the Bt strategy has become a success, other peptides, including those showing antimicrobial activities against bacteria and fungi are far from being utilized in agricultural practice. But there is an enormous potential: The review of Marcos et al. (2008) provides a comprehensive overview of recent progress using antimicrobial peptides (AMPs) from various sources in plants. The rapidly increasing number of reports demonstrates that AMPs are capable of strengthening plant health by expressing the respective heterologous gene in crop plants. Intriguingly, a recent paper demonstrates the considerable specificity of an antimicrobial peptide from insects, metchnikowin. This AMP not only distinguishes between bacteria and fungi but also within fungal phyla (Rahnamaeian et al., 2009). Thus, further investigation into AMPs' mode of action will greatly promote novel strategies to use them in future plant production measures. We will focus our discussion here on the potential of AMPs from insects with emphasis on our recent work on specificity of AMPs from various insect sources in controlling plant pathogenic fungi.

7.2 Insect Antimicrobial Peptides

To date approximately 900,000 insect species have been described which represent about 80% of all the world's species. The total number of insect species can only be estimated by former and current studies and is supposed to range between 10 and 30 million species. The reason for this incredible number of existing species is not the high rate of evolution of new species but rather the low extinction rate this animal group is suffering in comparison to others. Although insects are continuously exposed to potentially pathogenic microorganisms and parasites, they show only very rarely an infection. Insects lack adaptive immunity, i.e., the generation of highly specialized and systemic cells producing antibodies. Adaptive immunity is thought to have first arisen in jawed vertebrates. Like plants, insects exhibit the much more efficient and faster innate immunity (Otvos, 2000). There are two major types of insect immunity: the cell-free or humoral immunity and the hemocytic or cellular immunity. Antimicrobial peptides, which are part of humoral immunity, can be detected as early as 2 h after a septic injury in the insect hemolymph (Meister et al., 1997). There is a whole arsenal of antimicrobial peptides that is produced in insects challenged by microbes. The synergistic effect of various AMPs and their broad antimicrobial range may be the clue for the successful defense found in insects against microbial infections.

The most recent period of research on insect immunity began in 1980 when the first anti-bacterial factor, called cecropin, was isolated and purified from the silkmoth *Hyalophora cecropia* (Hultmark et al., 1980). Since then more than 50 AMPs have been isolated from insects and a total of more than 1,500 AMPs derived from microorganisms, plants, and animals have been reported in the literature to date. Antimicrobial peptides from insects have in general fewer than 50 amino acid residues and are cationic. They can be divided into several groups based on structural characteristics: (1) linear and amphipathic often α-helical AMPs; (2) linear peptides rich in certain amino acids, like Pro, Arg, or Trp; (3) AMPs with a single

Table 7.1 Pathogen resistance induced by insect antimicrobial peptides in plants

Peptide	Host	Pathogen(s)	References
Cecropin A	Rice (*O. sativa*)	*M. oryzae*	Coca et al. (2006)
SB-37 (Cecropin B derivate)	Potato (*S. tuberosum*)	*E. carotovora*	Acre et al. (1999)
	Tobacco (*N. tabacum*)	*P. syringae* pv. *tabaci*	Jaynes et al. (1993) and Huang et al. (1997)
Cecropin–melittin hybrids	Tobacco (*N. tabacum*)	*F. solani*	Yevtushenko et al. (2005)
	Potato (*S. tuberosum*)	*F. solani, P. cactorum, E. carotovora*	Osusky et al. (2000)
Sarcotoxin IA	Tobacco (*N. tabacum*)	*P. syringae* pv. *tabaci*, *E. carotovora* *R. solani*, *P. aphanideratum*, *P. nicotianae* pv. *nicotianae*	Ohshima et al. (1999) Mitsuhara et al. (2000)
	Tomato (*S. lycopersicum*)	*O. aegyptiaca*	Aly et al. (2006)
Attacin E	Pear (*Pyrus* ssp.) Apple (*Malus* ssp.)	*E. amylovora*	Kisung et al. (2002), Norelli et al. (1994), and Reynoird et al. (1999)
	Potato (*S. tuberosum*)	*E. carotovora* ssp. *atroseptica*	Acre et al. (1999)
Heliomycin Drosomycin	Tobacco (*N. tabacum*)	C. *nicotianae*	Banzet et al. (2002)
Gallerimycin	Tobacco (*N. tabacum*)	*E. cichoracearum*, *S. minor*	Langen et al. (2006)
Metchnikowin	Barley (*H. vulgare*)	*F. graminearum*, *B. graminis* f.sp. *hordei*	Rahnamaeian et al. (2009)

cyclic disulfide; (4) AMPs with several internal disulfides. In the following those insect AMPs are outlined that have been used for plant disease control from the 1980s till today. Table 7.1 shows a selection of reports on successful engineering of plant disease resistance by insect AMPs.

7.3 Cecropins

Cecropins are linear, amphipathic peptides of 35–39 amino acid residues derived from preproproteins of 62–64 residues. These preproproteins contain an amino-terminal signal peptide which targets them into the secretory pathway. Cecropins contain two distinct α-helical regions, a strongly basic N-terminal domain and a hydrophobic C-terminal helix, linked by a short, flexible hinge. Cecropins show post-translational amidation which prevents cleavage by carboxypeptidases and provides an additional bond for the formation of α-helices (Andreu and Rivas, 1998). The amidation of the C-terminus is a prerequisite for the full biological activity of this AMP group. More than 60 cecropin isoforms and cecropin-like sequences have been isolated from a wide variety of organisms ranging from insects to tunicates and mammals (Elkengren and Hultmark, 1999). Insect cecropins are almost exclusively generated by the Lepidoptera and Diptera orders and were first discovered in the silkmoth *H. cecropia*. The amphipathic properties of cecropins enable them to interact with the negatively charged components of the microbial envelope, such as lipopolysaccharides (LPS) and to penetrate the cell membrane. It is an accepted model that the interaction of cecropins with membrane lipids results in a disintegration of membrane structure by forming membrane pores that lead to an increase in plasma membrane permeability. This model is supported by the finding that there is a direct correlation between the cationic character of cecropins and their antimicrobial activity, as the less cationic cecropin D isolated from the cecropia silkmoth shows a lower antimicrobial activity compared to cecropin A and B, which have a higher positive net charge. Cecropins are active against a large variety of Gram-positive and Gram-negative bacteria as well as fungi. In general their activity against bacteria is higher than their anti-fungal potential (Elkengren and Hultmark, 1999). Plant and animal cells seem to be completely insensitive to cecropins. This selectivity of the cecropins can be explained by the different composition of cell membranes, like phosholipid composition, sterol content, membrane potential, or the presence of polyanions like LPS.

The expression of anti-fungal genes from plants leads only to moderate resistance against pathogens, most probably because plant pathogens have already developed tolerance to such plant-derived proteins. In contrast, heterologous expression of genes encoding antimicrobial peptides of animals, fungi, or bacteria in plants has been proven to confer high levels of protection and broad spectrum resistance against pathogens (Osusky et al., 2000).

To answer the question, whether cecropins are suitable candidates for the generation of disease-resistant crop plants, the antimicrobial activity of cecropins and their derivates against plant pathogenic bacteria and fungi was tested. In vitro

tests with cecropin B revealed a stronger anti-bacterial than anti-fungal activity of this AMP. The growth of plant pathogenic bacteria like *Pseudomonas syringae*, *Xanthomonas campestris*, or *Erwinia carotovora* was completely inhibited by concentrations of cecropin B between 5 and 15 μg/mL (1.3–3.9 μM). To inhibit plant pathogenic fungi like *Penicillium digitatum* or *Phytophthora infestans*, concentrations of 15.6 μM and higher were needed (Alan and Earle, 2002). In another study truncated variants of cecropin A were tested in vitro against several *Fusarium* species and other microbial plant pathogens like *P. infestans*, *Magnaporthe oryzae*, and *Botrytis cinerea*. Here concentrations between 6.5 and 90 μM of the cecropin A-derived peptides led to a total growth inhibition (Cavallerin et al., 1998). Rice plants transformed with a codon-optimized cecropin A gene exhibited enhanced resistance against the rice blast fungus *M. oryzae*. The anti-fungal activity of the synthetic cecropin A peptide was confirmed by in vitro inhibition tests with protein extracts prepared from leaves of transformed rice plants (Coca et al., 2006). Surprisingly, plants transformed with the cecropin B gene showed no resistance against plant pathogens. Allefs and coworkers (1995) generated potato lines expressing the cecropin B gene which were not resistant against *Erwinia* soft rot caused by *E. carotovora*. Furthermore, tobacco plants transformed with the cecropin B gene showed no resistance against *P. syringae* pv. *tabaci* (Hightower et al., 1994; Florack et al., 1995). The reason for these deflating findings is that the cecropin B peptides are highly susceptible to degradation by endogenous plant proteases (Mills et al., 1994). Therefore, cecropin B was modified and derivates like SB-37 were created to circumvent the degradation problem. SB-37 differs in only three amino acids from cecropin B, but these minor changes are sufficient to protect this cecropin B derivate from degradation by plant proteases. Potato plants expressing the SB-37 gene showed enhanced resistance against *E. carotovora* (Acre et al., 1999) and tobacco plants expressing SB-37 were resistant against *P. syringae* pv. *tabaci* (Jaynes et al., 1993; Huang et al., 1997).

Another successful strategy to generate pathogen-resistant crop plants is the combination of cecropin with other AMPs like melittin. Melittin is the major lytic component of the bee venom. It consists of 26 amino acids, with a predominantly hydrophobic N-terminus and amphipathic C-terminus and has excellent anti-bacterial properties; but its hemolytic activity makes this AMP unsuitable for expression in transgenic plants. The cecropin–melittin hybrid peptides that are only 11–28 amino acid residues long and combine the biologically active portions of the two parental peptides show a better protection against degradation by proteases and lack the undesirable toxic effects of melittin. The hybrid peptides were tested in vitro against several plant pathogenic bacteria and fungi. They exhibited a broad antimicrobial activity and inhibited the growth of the bacteria *P. syringae*, *Erwinia amylovora*, *Xanthomonas vesicatoria* (Ferre et al., 2006) and of oomycetes and fungal pathogens like *P. infestans*, *F. oxysporum*, or *M. oryzae* (Cavallerin et al., 1998).

Transgenic tobacco plants expressing a cecropin–melittin antimicrobial hybrid peptide of 29 amino acids were found to be resistant to *F. solani* and showed no toxic effect on plants (Yevtushenko et al., 2005). The cecropin–melittin hybrid construct CEMA conferred resistance against *F. solani*, *Phytophthora cactorum*, and

E. carotovora when expressed in transgenic potato plants (Osusky et al., 2000). In conclusion, cecropins and cecropin-derived antimicrobial peptides are promising candidates in engineering plant resistance against bacterial and/or fungal diseases.

7.4 Sarcotoxins

Sarcotoxins are antimicrobial peptides released into the hemolymph of larvae and nymphs of the flesh fly *Sarcophaga peregrina* upon mechanical injury and bacterial infection (Aly et al., 1999). Four sarcotoxin genes (IA to ID) arranged in a gene cluster have been identified in *S. peregrina* (Matsuyama and Natori, 1988). They encode precursors of the sarcotoxins produced in fat bodies with a length of 63 amino acids. The mature sarcotoxin peptides are 39 amino acids long and show a strong sequence homology to cecropins. Sarcotoxin peptides consist of two amphiphilic α-helical regions linked by a short hinge. The amidated N-terminus of sarcotoxins is hydrophilic while the C-terminus displays hydrophobic properties (Okada and Natori, 1985; Ohshima et al., 1999). Sarcotoxins are proposed to act as ionophores with the C-terminus penetrating the bacterial membrane while the N-terminus interacts with acidic phospholipids. Cholesterol that is present in eukaryotic membranes inhibits the activity of sarcotoxin which may be one reason why plant and animal cells are less sensitive to sarcotoxins in comparison to bacteria (Nakajima et al., 1987). Sarcotoxin IA was shown to disrupt the protein gradient and membrane potential of bacteria (Okada and Natori, 1985). Okemoto and coworkers (2002) generated truncated derivates of sarcotoxin IA and tested them for binding to LPS. They confirmed a binding of native sarcotoxin IA to the lipid A core of LPS and showed that the first two N-terminal amino acids, Gly and Trp, are essential for the LPS bond. The anti-bacterial activity of sarcotoxin IA is higher compared to cecropins and comparable with that of antibiotics. The minimal inhibitory concentration against *E. coli* ranges between 0.2 and 0.3 μM (Mitsuhara et al., 2000).

When the sarcotoxin IA gene was expressed in transgenic tobacco under control of the constitutive promoter CaMV35S, only faint accumulation of the respective peptide was observed (Okamoto et al., 1998). Therefore, different strategies were applied to enhance the expression level of the antimicrobial peptide. The fusion of sarcotoxin IA to GUS was found to increase the amount of transgenic protein in tobacco. Additionally, the sarcotoxin IA-GUS fusion protein contained a signal peptide sequence from pathogenesis-related gene PR1a of tobacco to ensure secretion of the mature protein into the inter-cellular space. The anti-bacterial potency of the fusion protein was confirmed by in vitro tests. Leaf extracts of transgenic tobacco lines strongly inhibited the growth of *E. coli* suspension cultures. Remarkably, the transgenic tobacco plants displayed an abnormal phenotype. The plants were shorter, the leaves were thicker with unusual shape and showed enhanced electrolyte leakage compared to leaves of untransformed plants suggesting that the strong transgene expression of sarcotoxin IA-GUS perturbs the plant membranes (Okamoto et al., 1998). Ohshima et al. (1999), in contrast, used the artificial E12Ω promoter

that includes tandem repeats of the 5′ enhancer sequence of the CaMV35S promoter and the Ω sequence from the tobacco mosaic virus (TMV) to drive the sarcotoxin IA gene for ectopic expression in tobacco. This sarcotoxin IA gene construct was fused to the signal sequence encoding region of PR1a. The resulting transgenic plants displayed normal phenotypes and enhanced resistance against the plant pathogens *P. syringae* pv. *tabaci* and *E. carotovora* ssp. *carotovora*. Plant cells were not harmed by sarcotoxin IA as cell suspension cultures of tobacco and rice showed no growth impairment when incubated with different concentrations of the peptide.

Sarcotoxin IA was also expressed in transgenic tobacco under control of the pathogen and salicylic acid-inducible tobacco PR1a promoter (Mitsuhara et al., 2000). The expression of the transgene could be increased up to tenfold by application of salicylic acid. Leaves of the transgenic plants were not only resistant to infection by bacterial plant pathogens but also to fungi like *Rhizoctonia solani*, *Pythium aphaniderатum*, and to the oomycete *Phytophthora nicotianae* var. *nicotianae*.

Not only microbes are affected by sarcotoxins. Aly and coworkers (2006) were able to enhance host resistance against *Orobanche aegyptiaca* by expression of sarcotoxin IA in tomato roots. The parasitic weed broomrape (*Orobanche* ssp.) is a chlorophyll-lacking obligate parasite that attacks roots of members of the *Solanaceae*, *Fabaceae*, and Compositae and thereby it causes severe losses in yield and quality of food and ornamental crops (Parker and Riches, 1993). Seeds of *Orobanche* germinate in response to a specific chemical germination signal from the host plant, indicating a close association between the parasite and its host. Transgenic tomato plants expressing sarcotoxin IA in the roots showed a normal development and growth and no toxic effect of the transgene on the host plant. *O. aegyptiaca* shoots attached to the transgenic roots turned necrotic and their development was abnormal. The transgenic plants challenged by *O. aegyptiaca* were significantly taller and contained more biomass than the attacked control plants. The mechanism that leads to enhanced resistance of the host plant against the parasitic weed by expression of antimicrobial sarcotoxin IA is still unknown.

7.5 Attacins

Attacins are glycine-rich immune proteins first isolated from pupae of the silkmoth *H. cecropia* in response to bacterial infection (Hultmark et al., 1983). Some attacins have been isolated also from *Drosophila melanogaster* (Dushay et al., 2000), the silkworm *Bombyx mori* (Taniai et al., 1996), the tobacco budworm *Heliothis virescens* (Ourth et al., 1994), the cabbage looper *Trichoplusia ni* (Kang et al., 1996), the Tsetse fly *Glossina morsitans* (Wang et al., 2008), and the house fly *Musca domestica* (Geng et al., 2004). The molecular weights of attacins range between 20 and 25 kDa and they can be divided into two groups, a basic and an acidic one, based on their amino acid composition and amino-terminal sequences. Attacins are active against some Gram-negative bacteria like *E. coli* but do not affect

Gram-positive bacteria or fungi. Carlsson et al. (1991) could show that the treatment of Gram-negative bacteria with attacins results in an increase in the permeability of the outer membrane, thus facilitating the access of lysozymes and cecropins into the bacterial cell. Additionally attacins lead to a specific inhibition of the synthesis of major outer membrane proteins in bacteria on a pre-translational level. In Tsetse flies attacins play a role in trypanosome resistance and in maintaining parasite numbers at homeostatic levels in infected insects (Wang et al., 2008).

Attacin genes were also successfully used for generating transgenic crop plants which showed enhanced resistance against plant pathogenic bacteria of the genus *Erwinia*. Norelli and coworkers (1994) transformed different apple and pear cultivars with the attacin E gene from *H. cecropia*. The resulting transgenic plants were resistant to fire blight caused by *E. amylovora* (Kisung et al., 2002; Norelli et al., 1994; Reynoird et al., 1999). The same gene resistance against *E. carotovora* ssp. *atroseptica* could be established in transgenic potato plants. The incidence of blackleg or soft rot disease could be reduced up to 83% in plants showing high levels of attacin E expression (Acre et al., 1999).

7.6 Defensins

Defensins represent the most wide-spread group of inducible antimicrobial peptides and they were mostly isolated from mollusk, acari, arachnids, insects, mammals, and plants. They are small, cationic, cysteine-rich peptides of 29–46 residues with three to six disulfide bonds. Insect defensins were first reported from cell cultures of the flesh fly *S. peregrina* and have been found in every insect species investigated to date. The peptide sequences are quite similar within an order of insects, but considerably different across the orders. In general the C-terminal domains are less conserved (Otvos, 2000). Most insect defensins contain three disulfide bonds of which two link an α-helix to a β-sheet. Only few insect defensins, like drosomycin, contain eight cysteine residues resulting in four disulfide bridges (Bulet and Stöcklin, 2005). Most defensins from insects contain a glycosylated threonine in the mid-chain position, but the presence of a sugar residue is not necessary for biological activity (Otvos, 2000). Amidation that is a prerequisite for the activity of cecropins can only rarely be observed in insect defensins. Insect defensins can be divided into two groups based on their activity: an anti-bacterial and an anti-fungal group. The anti-bacterial activity of the first group is restricted to Gram-positive bacteria; Gram-negative bacteria, filamentous fungi, and yeast in contrast are less sensitive to these defensins. Gram-positive bacteria are affected by minimal inhibitory concentrations often below the μM range and within minutes after application of the anti-bacterial peptide. In vitro studies revealed that the anti-bacterial activity decreases significantly in culture media under physiological conditions, so that one could question whether the anti-bacterial effect of defensins is really a physiological trait in insects (Bulet and Stöcklin, 2005). The mode of action of

anti-bacterial defensins is poorly understood. One prerequisite for the anti-bacterial activity is the positive net charge of the defensins. This facilitates the interaction with the acidic cell wall of the bacteria, which consists of multiple layers of peptidoglycan with teichoic acid polymers dispersed throughout. Cociancich and coworkers (2005) elucidated the action of defensin A isolated from the blowfly *Phormia terraenovae* on *Micrococcus luteus* cells. The defensin disrupts the permeability barrier of the cytoplasmic membrane most probably by forming pores by defensin oligomers and leads to a loss of cytoplasmic potassium, a partial depolarization of the inner membrane, a decrease in cytoplasmic ATP, and eventually an inhibition of respiration.

The anti-fungal group of insect defensins comprises drosomycin from the fruitfly *D. melanogaster*, heliomycin from the tobacco budworm *H. virescens*, gallerimycin from the greater wax moth *Galleria mellonella*, termicin from the termite *Pseudacanthotermes spiniger*, and Alo 13 from the harlequin beetle *Acrocinus longimanus*. Out of these defensins only termicin has also the potency to affect the growth of Gram-positive bacteria; the others are strictly antifungal and either inhibit spore germination or lead to hyphal perforation (Bulet and Stöcklin, 2005). Thevissen et al. (2004) showed that heliomycin interacts with the fungal glucosylceramides of *Pichia pastoris* and leads to enhanced membrane permeabilization. To date it is not known whether insect defensins also have intra-cellular targets like plant defensins that were shown to inhibit protein synthesis in eukaryotic and prokaryotic cell-free systems (Mendez et al., 1996).

Heliomycin, drosomycin, and gallerimycin have been used to establish resistance in transgenic tobacco against fungal pathogens (Banzet et al., 2002; Langen et al., 2006). Heliomycin and drosomycin both are 44 amino acid residues long and share significant structure homology. In vitro studies with recombinant peptides showed a strong anti-fungal effect of both defensin-like AMPs against several important crop plant pathogens like *Fusarium* ssp., *M. oryzae*, *R. solani*, *Sclerotinia sclerotiorum*, or *Septoria tritici* at concentrations as low as 10 μg/mL. Both AMPs were constitutively expressed in transgenic tobacco plants after fusing the genes encoding the mature peptides to a plant signal sequence resulting in an extra-cellular localization of the anti-fungal peptides. Treatment of *B. cinerea* spores with the anti-fungal peptides isolated from protein fractions of transgenic tobacco plants resulted in a swelling of the spores, release of cytoplasmic content, and inhibition of spore germination at 40 and 20 μg/ml for heliomycin and drosomycin, respectively, demonstrating that both AMPs were expressed and folded in their anti-fungal active conformation in tobacco. The inoculation of transgenic tobacco plants expressing either heliomycin or drosomycin with the pathogenic fungus *Cercospora nicotianae* resulted in a moderate reduced infection (up to 50%), compared to unmodified tobacco plants (Banzet et al., 2002).

Gallerimycin is a defensin-like peptide from *G. mellonella* of 76 amino acid residues, containing an N-terminal signal peptide of 20 amino acids which leads to an apoplastic localization of the mature peptide. Gallerimycin was isolated from a hemocyte cDNA library enriched with transcripts upregulated after immune

challenge via subtractive suppression hybridization (SSH) comparing the hemolymph of larvae injected with lipopolysaccharides with naïve larvae. Gallerimycin showed no effect in vitro on bacteria like *M. luteus*, *Bacillus subtilis*, or *E. coli* nor on the yeast *S. cerevisiae*. But it was active against the entomopathogenic fungus *Metarhizium anisopliae*, an important fungus for biological control of insect pests (Schuhmann et al., 2003). Langen and coworkers (2006) expressed gallerimycin in transgenic tobacco and tested the leaf sap and the inter-cellular washing fluid (IWF) against the biotrophic powdery mildew fungus *Erysiphe* (*syn. Golovinomyces*) *cichoracearum* and the necrotrophic *Sclerotinia minor*. They could demonstrate that germination of *E. cichoracearum* spores in the IWF of transgenic tobacco plants was inhibited up to 66%. The presence of anti-fungal activity in the IWF showed that gallerimycin was secreted to the plant apoplast and confirmed that the insect signal peptide of gallerimycin is functional *in planta*. The anti-fungal effect of gallerimycin was further confirmed when leaves of transgenic tobacco plants inoculated with *E. cichoracearum* or *S. minor* showed less symptoms than the leaves of untransformed plants.

In conclusion, insect defensins display a pool of potential candidates for generating transgenic crop plants with a potent resistance against either bacterial or fungal plant pathogens.

7.7 Metchnikowin

Metchnikowin is a 26-amino acid residue proline-rich linear peptide synthesized in the fat bodies of *D. melanogaster* as a 52-amino acid prepropeptide upon microbial challenges. According to original studies, the peptide inhibits the growth of Gram-positive bacteria and the ascomycete fungus *Neurospora crassa* (Levashina et al., 1995). Chemically synthesized mature 26-amino acid metchnikowin inhibited macroconidia germination of the ascomycete *Fusarium graminearum* with an IC_{50} value of 1 μM. The prepropeptide gene was used for plant transformation in order to target the mature peptide into plant apoplastic space (Rahnamaeian et al., 2009). This strategy had the advantage of minimizing post-translational degradation by intra-cellular proteases. IWF from transgenic barley plants expressing the prepropeptide of metchnikowin inhibited hyphal growth of *F. graminearum* when compared with IWF from the untransformed barley. Consistent with in vitro inhibition of *F. graminearum* by the synthetic metchnikowin, transgenic barley plants showed decreased *Fusarium* head blight and root rot damages.

Additional experiments showed that metchnikowin-expressing barley was also more resistant to the biotrophic barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*, Fig. 7.1). Germination of *B. graminis* conidia on the leaf surface of transgenic plants was not inhibited, but fungal penetration from the appressorial germ tube into the leaf epidermis was strongly reduced. Interestingly, microscopic analysis of transgenic plants showed higher frequencies of typical cellular defense responses, for example death of attacked epidermal cells (the so-called

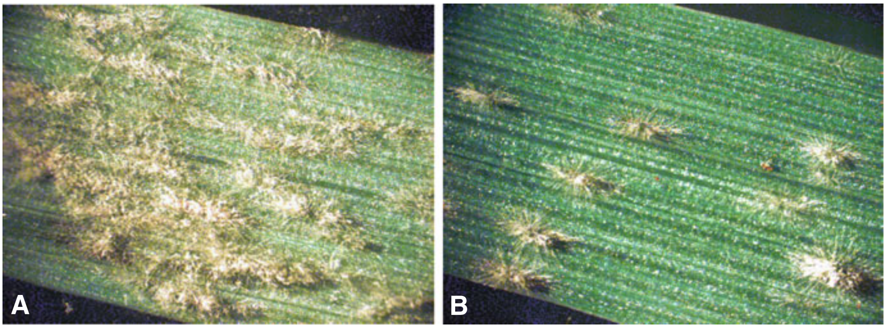


Fig. 7.1 Reduced powdery mildew infections on barley plants expressing the antimicrobial peptide metchnikowin. **a**) Heavily infected barley first leaf segment (cv. Golden Promise) at 5 days of inoculation with 5 conidia per mm^2 of *Blumeria graminis* f.sp. *hordei* using air current dispersal in an inoculation pillar. **b**) Powdery mildew colony formation on metchnikowin-expressing barley is significantly reduced. Macroscopic evaluation of reduced infection was confirmed by microscopic analysis at early infection (*2 days after infection*) which showed higher frequencies of cell wall appositions (CWA) and hypersensitive responses (HR, not shown). For details see Rahnamaeian et al. (2009)

hypersensitive response [HR]) and cell wall appositions (CWA) underneath attempted penetration sites. Hence, in an unanticipated way, heterologously expressed metchnikowin activated the plants' "natural" defense system in epidermal cells, which consequently led to a reduced number of functional fungal haustoria, reduced colonization, and eventually to disease resistance. One explanation for the action of metchnikowin is that its presence in the apoplastic leaf space may weaken the fitness of the attacking fungus and thereby disturb the pathogenic fungus' essential capability to actively suppress plant defense. Effective suppression of the host plants' innate immunity system is a prerequisite for a successful fungal infection, for a compatible interaction, and eventually for the outbreak of the disease (Hückelhoven and Kogel, 1998; Kogel et al., 2006). Since defense suppression is thought to be brought about by effector molecules frequently secreted by microbes, including plant pathogenic fungi (Van der Hoorn and Kamoun, 2008), it has been suggested that the fungal secretory pathway is perturbed by metchnikowin (Rahnamaeian et al., 2009). Interestingly and in accordance with this hypothesis, barley plants, that accumulate metchnikowin in the apoplastic space, are also more resistant to spot blotch disease caused by the hemibiotrophic fungus *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus*; Kumar, unpublished; Fig. 7.2). Leaves from metchnikowin expressing plants form untypical large cell wall appositions at sites of attempted infection in response to attack by germinating spores (Fig. 7.3) while untransformed plants show rather small CWA in only low frequencies.

Metchnikowin was likewise effective against ascomycota from the genus *Fusarium,* against *B. graminis* and *C. sativus* with LD_{50} of approximately 1 mM. In clear contrast, the basidiomycetes *Piriformospora indica* and *R. solani* were insensitive to the same metchnikowin concentration. LD_{50} for the inhibition of

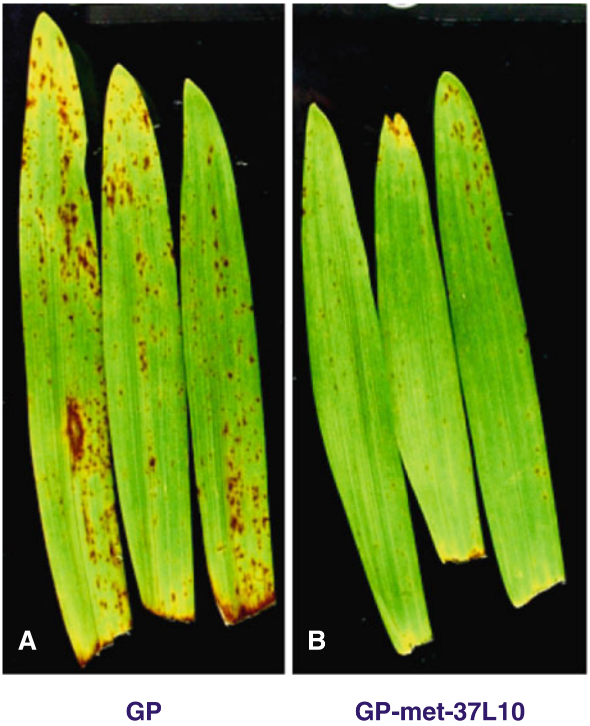


Fig. 7.2 Reduced spot blotch infections on barley plants expressing the antimicrobial peptide metchnikowin.
a) Infected barley first leaf segment (cv. Ingrid) at 4 days after inoculation with 10 conidia per mm^2 of *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus*).
b) Spot blotch symptoms on metchnikowin-expressing barley are significantly reduced (Kumar et al., unpublished)

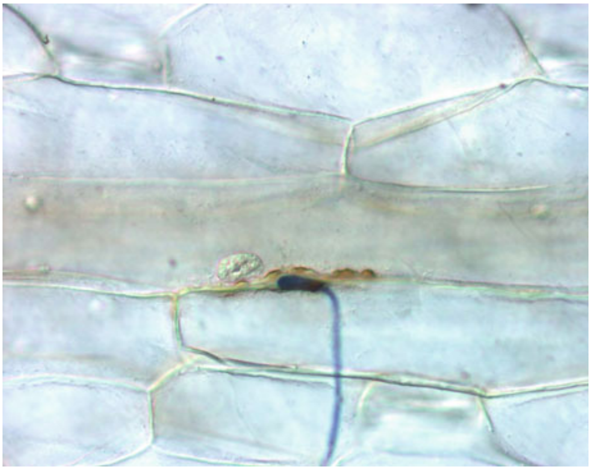


Fig. 7.3 Micrograph of a cytological response of barley expressing the antimicrobial peptide metchnikowin. A huge cell wall apposition (*papilla*) is formed beneath the attacking hypha of *Bipolaris sorokiniana* (syn. *Cochliobolus sativus*). Phenolic compounds accumulate in and around the papilla observed as autofluorescence under UV light excitation (Kumar et al., unpublished)

spore germination of these basidiomycetes was higher than 50 μM. Equally, hyphal protoplasts exhibited differential sensitivities in the same orders of magnitude further suggesting that metchnikowin is differentially active on ascomycetes and basidiomycetes (Rahnamaeian et al., 2009).

7.8 Future Prospects

7.8.1 Rational Design of AMPs

As described earlier in this chapter some natural AMPs show undesirable properties such as non-specific toxicity, low stability, or poor bioavailability. For application in plant biotechnology a toxic effect on plant cells and/or a fast degradation of the AMPs in the cellular milieu are unwanted. Therefore, a holistic approach to enhance their stability, potency, and specificity towards certain microbes is aspired to. The short sequence length of AMPs and their corresponding genes facilitate the design of synthetic genes and the chemical synthesis of synthetic AMPs. Rational design of AMPs can bring enhanced specificity against pathogens, reduced toxicity against plant and animal cells, higher stability, and a modulation of the host spectrum of action. One example for successfully designed synthetic AMPs are the cecropin–melittin hybrids that contain the antimicrobial active domains of the parent peptides but lack the undesirable hemolytic effect of melittin (Jacobi et al., 2000). Furthermore, cecropin–melittin hybrids show a high stability in plant extracts, a feature that is missing in cecropins, thus making the hybrids – in contrast to the natural AMPs – suitable for the generation of pathogen-resistant crop plants. The peptide D4E1, a synthetic homolog of cecropin B, inhibits spore germination of fungal pathogens much more efficiently than the parent peptide but does not show any effect on the germination of pollen and seed of pine, spruce, or aspen as was found for the natural cecropin B.

The activity of some AMPs has been enhanced by sequence modifications that lead to an increase in positive net charge or higher amphipathicity compared to those of the parent AMP. By this means the activity against bacteria can be boosted markedly, as the interaction between the negatively charged outer membrane of the bacteria and the positively charged AMP would be enhanced.

Cyclization of peptides can increase their stability and specificity. This modification is particularly interesting for the biotechnological production of peptides that are used as phytosanitary products for field or post-harvest treatments (Marcos et al., 2008).

The modern methods of synthetic combinatorial chemistry should be applied to optimize insect-derived AMPs. Defined libraries of peptides that contain a core amino acid sequence with the antimicrobial activity of the natural AMPs can be generated and screened in large scale for in vitro activity against certain microbial pathogens, plant and animal cells and for stability in the presence of proteases or plant extracts. Such a strategy will lead to the identification of synthetic peptides

that are much more potent and stable than the parent AMPs while lacking undesired toxic effects (Marcos et al., 2008).

7.8.2 Directed Discovery of Specific Insect AMPs

Most insect AMPs show a broad activity against bacterial and/or fungal pathogens, but AMPs that are active against specific pathogens are often more desirable. To find these specialized AMPs, different approaches can be followed (Vilcinskas and Gross, 2005). As mentioned before, SSH was applied to identify genes that were up-regulated in the larvae of the greater wax moth (*G. mellonella*) upon injection of LPS and led to the discovery of the anti-fungal AMP gallerimycin (Schuhmann et al., 2003). To identify AMPs that are specific for a given microbe, a directed SSH approach could be conducted, where the target organism is applied to insects and genes are identified that are up-regulated only in response to this individual pathogen.

Phage-display techniques have already successfully been used to identify peptides that interact either with intra-cellular biomolecules or spores of certain plant pathogens. Liu and coworkers (2005) inhibited the successful establishment of cyst nematodes in potato by overexpression of the synthetic peptide ACHE-I-7.1. This peptide was found by a phage display approach. It inhibits acetylcolinesterase and thereby disrupts chemosensing in nematodes. Phage display also led to the isolation of randomly generated octamers with affinity to zoospores of *Phytophthora capsici* (Bishop-Hurley et al., 2002). Potato plants expressing these peptides were significantly less susceptible to root infection with *P. capsici* than untransformed plants (Fang et al., 2006).

The yeast two-hybrid system is another method to identify peptides that are specifically active against single pathogens. Lopez-Ochoa and coworkers (2006) isolated several peptides of around 20 residues in length that interacted in vivo with a replication protein of the tomato golden mosaic geminivirus (TGMV) and successfully inhibited viral DNA accumulation in tobacco protoplasts. The yeast two-hybrid system was also used to identify peptides that interact with nucleocapsid proteins of the tomato spotted wilt virus (TSWV). The peptide T220/248 consisting of 29 amino acid residues was identified to interact not only with the nucleocapsid of TSWV but also with that of other tospoviruses. Transgenic tobacco plants expressing T220/248 showed strong resistance when challenged with TSWV or other tospoviruses (Rudolph et al., 2003).

Some research was also directed to identify genes of biocontrol agents like *Bacillus* species that encode AMPs responsible for the control of plant pathogenic fungi and bacteria. Mutant strains deficient for the production of certain AMPs were implied in infection studies and led to the identification of peptides that are active against certain pathogens, like surfactin or mycosubtilin of *B. subtilis* that inhibit *P. syringae* or fungal species of the genus *Phytium*, respectively (Montesinos, 2007). Insect species exhibiting prominent resistance against microbial infection could also be screened to identify AMPs that are responsible for the defense against

pathogenic fungi or bacteria (Altincicek and Vilcinskas, 2008). For the future these methods should be suitable for the discovery of antimicrobial peptides from insects that show a specific activity against individual plant pathogens and lack undesirable side effects on other microbes like beneficial symbionts or mycorrhiza.

7.8.3 Inducible and Tissue-Specific Expression of Insect AMPs

The expression of transgenes in plants is a complex and much discussed issue. Particularly with regard to the commercialization of transgenic plants many aspects have to be taken into consideration.

For research purposes, a constitutive expression of the transgene is often chosen with the transferred gene driven by the Cauliflower Mosaic Virus 35S (CaMV 35S) promoter, or the plant ubiquitin promoter. But for commercial applications this is often not suitable. Many critics and antagonists of genetic engineering and transgenic plants argue that the constitutive expression of transgenes increases the risk of resistance formation in target organisms or that the constitutive expression leads to an unwanted systemic accumulation of the transgene in crop plants that might cause allergies or have negative impacts on ecosystems and non-target flora and fauna (National Research Council, 2002). The constitutive expression of the transgene might also have an impact on the whole expression profile in the transgenic crop plant. Campo and coworkers (2008) showed by a PCR-SSH approach that the constitutive expression of cecropin A with an ER-retention signal peptide altered the transcription of at least 168 genes in transgenic rice plants. Remarkably many of these genes were involved in the protection of plant cells against oxidative stress or protein synthesis, folding and stabilization. Additionally, the constitutive expression of a transgene in plants can lead to unwanted loss of energy resources due to unnecessary accumulation of transgene products thereby leading to yield reduction (Altpeter et al., 2005). To circumvent such unwanted side effects, a more controlled expression of the transgene is desirable. This can be achieved either by choosing inducible promoters that allow the expression of the transgene on demand, for example only upon pathogen attack, or by tissue-specific expression of the transgene. Pathogen-induced promoters have already been employed to express AMPs in transgenic plants. A modified PR1a promoter from tobacco was used to generate transgenic tobacco plants that express sarcotoxin IA in response to bacterial and fungal infection. The level of resistance in these transgenic plants against *E. carotovora* ssp, *carotovora*, *P. syringae*, *R. solani*, and *Pythium aphanidermatum* correlated with the expression level of the AMP (Mitsuhara et al., 2000). The win3.12T promoter from poplar that is responsive to fungal infection was fused to genes coding for a cecropin–melittin hybrid or amphibian antimicrobial peptides. The resulting constructs were transferred into tobacco plants and led to enhanced resistance to different plant pathogens like *Fusarium* ssp., *Alternaria alternata*, or *Pectobacterium carotovorum* (Yevtushenko et al., 2005; Yevtushenko and Misra, 2007). Yi and coworkers (2004) could even demonstrate that plants expressing an AMP under control of a pathogen-induced promoter show a more reliable pathogen resistance

than plants expressing the same AMP constitutively. Beside pathogen-induced promoters also chemically induced ones can be employed so that the expression of the AMP can be directed by the application of certain chemicals. Progress is being made on expression systems for transgenic plants that use promoters responsive to for example ethanol, tetracycline, copper, or hormones (Christou et al., 2006).

But not only the temporal but also the spatial control of the expression of the transgene is desired. For this purpose a number of tissue-specific promoters are available. For phloem-feeding insects for example the root phloem-specific promoter AAP3 from *Arabidopsis thaliana*, as well as the phloem-specific pumpkin promoter PP2, or the rice sucrose synthase promoter Rss can be applied to drive AMP expression (Christou et al., 2006). To protect the crop after harvest from pathogen infection, the expression of the AMP can be restricted to the seeds or fruits as is observed for many intrinsic plant antimicrobial peptides. Fungi or insects that first attack the plant epidermis could be combated in transgenic plants where the AMP is driven by an epidermis-specific promoter. An example is the synthetic GstA1i promoter that is a fusion product of fragments of three different promoters that drive genes specifically in the wheat epidermis (Altpeter et al., 2005).

The transgenic expression of small AMPs can particularly implicate the potential problem of low stability due to small size and susceptibility to protease degradation *in planta*. Beside the rational design of AMPs to enhance their stability, the direction of the expressed peptide in the transgenic plant to locations where protease activity is presumed to be low, for example the apoplast, might also help to prevent AMP degradation (Marcos et al., 2008). An additional strategy to stabilize the AMPs *in planta* might be the simultaneous co-expression and co-secretion of protease inhibitors (Vilcinskas and Gross, 2005). This approach has successfully been applied to stabilize monoclonal antibodies expressed in tobacco roots by co-secretion of a Bowman-Birk protease inhibitor (Komarnytsky et al., 2006). The stability of the expressed AMPs can also be increased by mimicking natural plant systems for the delivery of small-sized peptides. Jones and coworkers (2004) were able to express the peptide Pep11 in tomato by substitution of systemin in prosystemin with pep11. Leaflets of the resulting transgenic tomato plants showed significantly reduced lesion sizes after inoculation with *P. infestans*.

A critical point which is one reason for the low acceptance of transgenic crops by the general public is the risk of cross-pollination between the transgenic crop plant and conventional crop or closely related weed plants leading to undesired pollen-mediated spreading of the transgene throughout the ecosystem. Currently the integration of the transgene into the chloroplast genome instead of the nuclear genome is the favored method to prevent the distribution of a transgene via pollen, as the plastid genome is inherited maternally. The antimicrobial peptide MSI-99, an analog of magainin 2, which is found in the skin of the African clawed frog, *Xenopus laevis*, has already successfully been transformed into the chloroplast genome of tobacco plants. The transgenic plants exhibited strong resistance against *P. syringae* pv *tabaci* (DeGray et al., 2001).

7.8.4 Fusion of AMPs and Pathogen-Specific Antibodies

Genetic engineering is one extra tool to generate plants with enhanced resistance against diseases and pests. A rather sophisticated approach is the expression of recombinant antibodies in plants that are directed to the outer structure of microbial pathogens or to essential components of the infection machinery and thus disrupt their infection cycle.

For the production of the recombinant antibodies different technologies are applicable. The hybridoma technology (Koehler and Milstein, 1975) can be used to identify full-length monoclonal antibodies that bind specifically to a target antigen. By default mice are injected with the pathogen of interest or parts of it like cell wall fragments. B-lymphocytes isolated from the treated mice are then fused with immortalized myeloma cells under selective conditions to establish a hybridoma line. Hybridomas that produce antibodies with affinity to the pathogen-derived antigen are selected, and their antibody-mRNA is isolated and transcribed into cDNA. Subsequently the cDNA can be used to design derivates of the full-size antibody like single-chain variable fragment (scFv), bispecific scFv, minibodies, diabodies, and fab fragments. The drawbacks of hybridoma technology are the high costs, the need of animal cell cultures and know-how to generate monoclonal antibodies by this method.

A powerful alternative to the hybridoma technology is phage display (Barbas et al., 1991). The standard procedure is to isolate mRNA from B-lymphocytes of a donor animal immunized with the pathogen of interest. Then polymerase chain reaction (PCR) is deployed to amplify the genes of the heavy (V_H) and the light (V_L) chain of the variable region of the polyclonal antibodies. The fragments of the two libraries are inserted into the genome of phage M13 by fusion to a truncated version of its minor coat protein (pIII). The fusion proteins are displayed on the surface of the mature phage while the genetic information of the corresponding V_H and V_L is contained inside the particle. That leads to the generation of a highly variable phage library with a nearly unlimited number of V_H and V_L recombinations. The phages are then subjected to panning, a simple in vitro selection procedure where phages that display antibody fragments binding to the pathogen-derived antigen are enriched. Therefore, phage display is a technique that allows the generation and isolation of highly specific antibodies in a time-saving and speedy manner.

Most of the antibodies that are expressed in plants to induce disease resistance are active against viruses, while only a few studies have been engaged in the resistance of plants against fungi or nematodes (for a review see Nölke et al., 2004). The antigens that served as targets for the recombinant antibodies were either viral coat proteins or secreted proteins of the microbial pathogens. Peschen and coworkers (2004) first described a promising new technology where antibody fragments (scFv) are combined with AMPs to generate a highly specific and effective pathogen resistance in plants. These researchers generated scFv fragments from chicken monoclonal antibodies immunized with cell wall-bound proteins, mycelium surface proteins, and germinated spores of *F. graminearum*.

The scFv CWP2 was isolated via phage display and showed a strong affinity to cell wall proteins in ELISA tests. Subsequently CWP2 was fused to three different AFPs, a hyphal wall-degrading wheat class I chitinase (Chi), an AFP from *Raphanus sativus* (RS), and an AFP from *Aspergillus giganteus* (AG). In vitro studies with the three fusion constructs revealed that CWP2 was essential for the anti-fungal effect of the AFPs as fusion-proteins of Chi, RS, or AG with a non-fungal antibody had no effect on the growth of *F. graminearum* or *F. oxysporum*. *Agrobacterium tumefaciens*-mediated transformation was employed to integrate the three constructs into the *A. thaliana* genome, and the resulting transformants were tested for resistance against *Fusarium oxysporum* pv. *matthiolae*. All three AFP-CWP2 combinations led to a significant reduction of disease symptoms up to 100%, and the protection against *F. oxysporum* pv. *matthiolae* was more efficient in plants expressing the fusion construct than in plants transformed either with CWP2 or one of the AFPs alone. Successive studies confirmed the efficiency of the fusion-construct AG-CWP2 against *Fusarium*. Wheat plants expressing AG-CWP2 displayed a significantly enhanced resistance when challenged with the FHB pathogen *Fusarium asiaticum*. In transgenic lines the initial infection and the spreading of the pathogen from spikelet to spikelet were reduced. Analyses of yield parameters revealed that plants expressing the antibody fusion produced up to 145% more grains than non-transgenic wheat plants after inoculation with *F. asiaticum* (Li et al., 2008).

This novel strategy has several advantages: First, the antibody directs the AMP to the target pathogen thereby generating highly localized toxic spots. It can be assumed that already comparably low doses of AMP are efficient. Thus, precise direction of the AMP towards the pathogen confers disease resistance at low expression levels of the transgene. In some wheat lines that expressed AMP-CWP2 fusion constructs and displayed significantly enhanced resistance against *F. graminearum*, the transgene could not be detected by Western blot (Jansen et al., unpublished). A second major advantage of the technology is that the additional specificity generated by the antibody means that only the target pathogen is affected by the AMP as demonstrated by Peschen and coworkers (2004). Such specificity was demonstrated by the authors when *Fusarium* species were inhibited by the AFP-CWP2 constructs while other fungal pathogens like *S. sclerotiorum* remained unaffected. Hence, the antibody-AMP fusion strategy might have the potential to become a can provide a precise tailored tool to combat devastating pathogens. At the same time beneficial microbes, for example mycorrhizal fungi or plant growth-promoting bacteria, can remain undisturbed. Since it is known that the latter microbes contribute to plant health and stable yields, the antibody-AMP fusion strategy could form a natural "plant interactome" and thus support sustainable production systems. In this way, the new strategy creates added value and may have clear advantages over chemical or even "biological" protection measures.

Together these advantages of AMPs make them a rational alternative or a supplementary measure for sustainable plant-production strategies.

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Chapter 8
Protection of Crops Against Insect Pests Using RNA Interference

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Abstract RNA interference (RNAi) caused by exogenous double-stranded RNA (dsRNA) has developed into a powerful technique for down-regulating gene expression in a wide range of organisms, following its discovery in the nematode *Caenorhabditis elegans*. Not only does import of dsRNA into cells to produce small interfering RNAs (siRNAs) take place readily in *C. elegans*, but also systemic RNAi effects can be shown to occur, which persist over distinct developmental stages. These effects are mediated by amplification of siRNAs and export to other cells in the organism. However, the sensitivity to RNAi in *C. elegans*, where specific genes can be down-regulated by feeding dsRNA, or even by soaking nematodes in dsRNA solutions, have not been duplicated in other organisms, nor have systemic and persistent RNAi effects been widely observed. Uptake of dsRNA was limited in the model insect species *Drosophila melanogaster* (fruit fly), and no evidence of systemic and persistent RNAi effects was observed. However, more recent research has shown that full RNAi effects can occur in insects, although they are variable from species to species. Down-regulation of gene expression through delivery of dsRNA to insects can cause mortality, through a range of altered phenotypes, such as interference with developmental processes, or metabolism, or responses to the environment, and systemic and persistent RNAi effects have been reported, most notably in the beetle *Tribolium confusum*. Whereas injection of dsRNA remains the method of choice for delivery to insects, in some species feeding dsRNA has also been shown to produce RNAi effects. Further, expression of dsRNAs directed against insect genes in transgenic plants has been shown to result in RNAi effects and to afford protection against insect herbivores. This technology has the potential to be the basis of a new generation of pest-resistant GM crops, complementing existing technologies, but further development to improve efficacy of protection, and range of species affected, will be necessary.

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8.1 Introduction

Insect-resistant transgenic crops expressing *Bacillus thuringiensis* (Bt) toxins are widely used in global agriculture, and, despite the controversy surrounding the introduction of genetically modified plants into the field, must be counted as one of the major successes of the "first generation" of plant genetic engineering (Toenniessen et al., 2003). However, this technology has only been deployed commercially to protect crops against lepidopteran and coleopteran pests (caterpillars of moths and butterflies, beetles), excluding many important pest species. In some cases, such as dipteran pests (flies), effective Bt toxins are available, but technical problems have prevented transgenic plants protected against them from being produced; in other cases, such as hemipteran sap-sucking pests (plant bugs, aphids, etc.), no Bt toxin with adequate insecticidal effects to give protection has been found. There is still a need for novel strategies which provide crop protection against insect pests (see Gatehouse, 2008). Suppression of expression of specific gene(s) in the pest by an RNA interference (RNAi) effect, through a plant-delivered RNA, offers the possibility of effective protection against any species, since genes necessary for survival, growth, development, reproduction, or feeding success can be targeted. The recent appearance of two reports on protection of plants against insect pests, by endogenous expression of RNA corresponding in sequence to pest genes, showed the feasibility of this technique (Mao et al., 2007; Baum et al., 2007a). This review will provide a summary of the background to the technology, discussing criteria which must be met in order for the strategy to work, and will summarise work to date in this area.

8.2 Regulation of Gene Expression by Small Cytoplasmic RNAs

The control of gene expression in eukaryotes can involve many different mechanisms, occurring at different stages of the processes of transcription and translation. Although primary control of expression is exerted at the transcriptional level, in the nucleus, post-transcriptional control due to mechanisms operating in the cytoplasm also plays a significant role in determining how much of a specific gene product is synthesised in the cell. These cytoplasmic post-transcriptional control mechanisms include regulation of translation, RNA localisation to specific parts of the cell, and RNA degradation. The involvement of small RNA molecules in both regulation of translation and degradation of specific RNAs has been a relatively recent discovery, but is emerging as a major factor in many examples of cell differentiation during development, and in defence against viral infection (for a recent review giving a historical perspective on these small RNAs and their roles in control of gene expression, see van den Berg et al., 2008.)

The active forms of small, regulatory RNA molecules in the cytoplasm are single stranded, and vary in length from 20 to 26 nt. They are conventionally divided into two functional types; micro RNAs (miRNAs), which act as transcriptional repressors, and short interfering RNAs (siRNAs), which cause degradation of specific RNAs. To show activity, both types of small RNAs must be bound to a multi-domain protein called Argonaute, in an RNA-induced silencing complex (RISC). Argonaute is an essential component of the RISC complex, but other proteins which act as modulators of the activity can also be associated. Both miRNAs and siRNAs are single stranded, and interact with target mRNA sequences by base pairing, with a specificity determined by complementarity of nucleotide sequences with their targets. miRNAs in RISC complexes bind to the 3′ untranslated regions (UTRs) of mRNA molecules, but an invariable feature of this interaction is that base pairing is not perfect throughout the entire length of the miRNA, with an unpaired region in the centre of the complex, and a minimum of six perfect matches over nt 2–7 of the miRNA. Once two or more RISC complexes are bound to distinct sites in the 3′ end of an mRNA, translation is inhibited, through a mechanism that remains to be fully elucidated (for further details of mechanisms involved in post-translational control of gene expression by miRNA, and determination of target sequences, see the review by Bartel, 2009). siRNAs in RISC complexes also bind to target RNAs, although this can occur anywhere within the sequence, and not just in the 3′ UTR. In contrast to miRNA binding, the base pairing between siRNA and its target is perfect throughout the length of the siRNA, except for a possible mismatch of 1–2 bases at the 3′ end of the siRNA. This perfect base pairing induces the Argonaute protein to cleave the target RNA at a position corresponding to nt 10 and 11 of the siRNA. The cleaved target mRNA fragments are released from the RISC complex and are subsequently degraded by cytoplasmic nucleases. The basic mechanisms of miRNA and siRNA activity are thus similar, although the end results of their action differ. The intra-cellular processes leading to RNA interference (RNAi) are shown in Fig. 8.1.

The genesis of RISC complexes containing siRNAs and miRNAs, which follow different converging pathways, is important in understanding how RNAi-mediated effects are produced. miRNAs are produced as precursors in the nucleus, which are exported to the cytoplasm through the nuclear export receptor exportin 5 (Yi et al., 2003). The exported precursor is a "hairpin" single strand of RNA, approximately 70 nt, folded back on itself, so that it contains a double-stranded "stem" (with an imperfect match between the two strands) and a single-stranded "loop". In the cytoplasm, a multi-domain protein termed Dicer cleaves this precursor into a double-stranded RNA of the final length of miRNA, with a single base 3′ overhang on each strand, and 5′ phosphorylation. The strand which will hybridise to the target mRNA (the guide strand) is then incorporated into the RISC complex, and the other strand is assumed to be degraded. The mechanism for strand selection in miRNAs is not fully understood, and in many cases both strands have been shown to be functional.

In contrast to miRNAs, which are the products of endogenous genes, siRNAs are produced from RNAs that are usually exogenous in origin. Double-stranded RNA

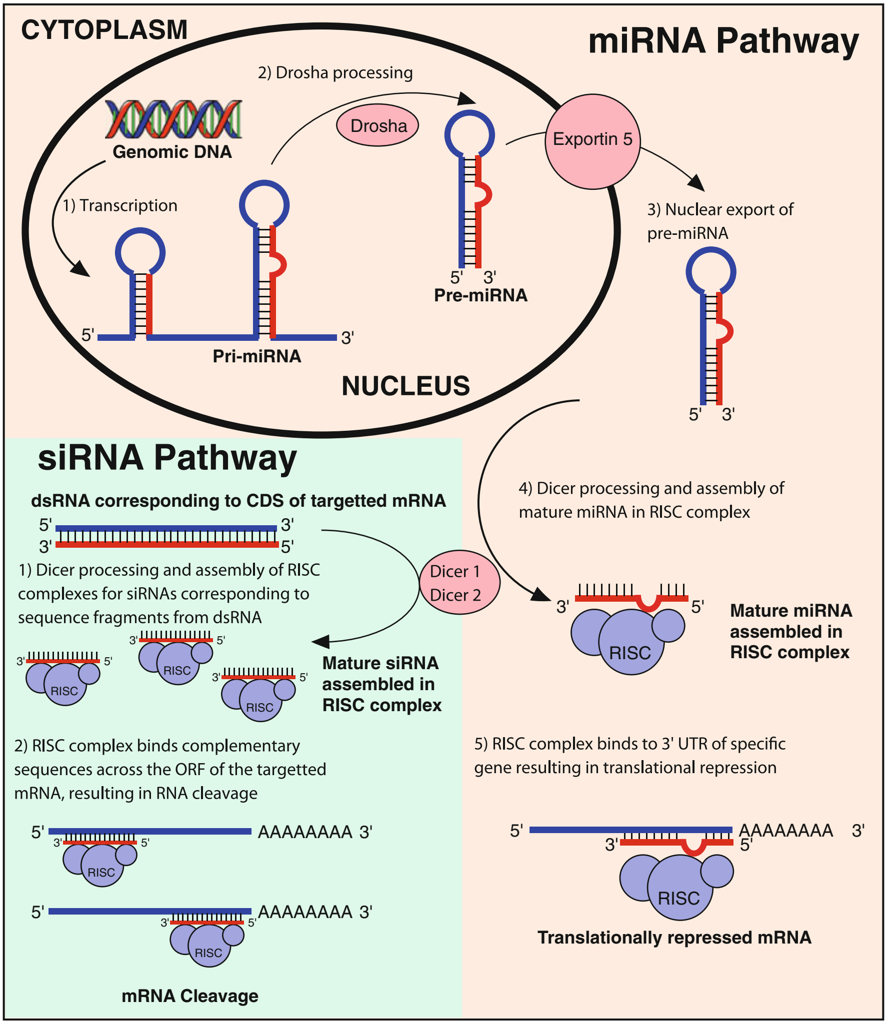


Fig. 8.1 Intra-cellular mechanism of generation and action of siRNAs and miRNAs. siRNAs can also be generated from a transgene construct inserted into the nuclear genome (see Section 8.4) by a mechanism similar to miRNA generation; in this case the hairpin RNA produced by transcription of the gene construct contains an inverted repeat which is a perfect match to part or all of the coding sequence of the target mRNA. These hairpin mRNAs are then processed by Dicer to generate siRNAs

molecules introduced into the cytoplasm are cleaved by the Dicer protein into multiple fragments, of the final length for siRNAs, with the single base 3′ overhang and 5′ phosphorylation noted above. Single strands from these double-stranded fragments are then incorporated into RISC complexes. In fruit fly (*Drosophila melanogaster*) the siRNA strand that is incorporated is determined by the activity of a protein sensor designated R2D2, which effectively carries out strand selection based on relative energies of unwinding the "ends" of the small dsRNA (Tomari et al., 2004).

The siRNA strand selection protein R2D2 is a necessary component for formation of RISC complexes in *D. melanogaster* (Pham and Sontheimer, 2005), but proteins similar to R2D2 are restricted to flies (Diptera), and possibly other insects, so the presence of R2D2 is not a general requirement for siRNA activity. Although siRNAs are normally produced as a result of the presence of dsRNA in the cytoplasm, siRNAs can also be derived from precursors exported from the nucleus like miRNAs, if suitable engineered gene constructs are introduced into the genome. These transgene constructs must contain a suitable perfect inverted repeat to produce a "hairpin" precursor similar to a "native" miRNA. Although introduction of transgene constructs demonstrates that it would be possible for the cell to produce siRNAs for endogenous genes, this seems to occur comparatively rarely in vivo, although some miRNAs have been shown to cause mRNA degradation as well as inhibit translation (Bagga et al., 2005).

The cytoplasmic proteins essential for RISC complexes to be formed are Dicer and Argonaute, which are found throughout the eukaryota, with multiple isoforms of one or both of them present in most organisms. siRNAs are thus considered to be a basic universal defence mechanism against viruses and transposons that generate dsRNAs during their replication or propagation. Mutations in the genes encoding Dicer or Argonaute leads to increased accumulation of transposon transcripts in *Drosophila* (Chung et al., 2008), and the increased susceptibility to RNA viruses in plants containing mutations in genes encoding Dicer and RISC complex components is well established. The older method for inducing gene silencing, anti-sense RNA, is actually an inducer of siRNA activity, via the formation of cytoplasmic dsRNA; the "antisense" RNA produced by an introduced gene construct forms a dsRNA with the native "sense" mRNA, and this dsRNA is then acted on by Dicer to generate siRNAs. miRNAs are much more variable between different organisms; their role as modulators of gene expression is consistent with this variability. The bases of miRNA targeting, and the mechanisms by which miRNAs regulate mRNA translation are still only partially understood, and as a result practical applications for this class of small RNAs are yet to be developed. Therefore, this chapter will focus on siRNAs, although potential "crossover" between siRNA and miRNA activities (Doench et al., 2003) may play a role in practical applications of RNA interference.

8.3 RNA Interference and Cellular Transport Mechanisms for RNA Import and Export – Systemic Effects

In order to generate specific gene silencing by exogenous RNA, a mechanism by which RNA can be transported into cells is necessary. RNA viruses have specific mechanisms for introducing their genetic material into cells, which avoid exposure of "naked" RNA to the extra-cellular medium, but studies in the nematode *Caenorhabditis elegans* have shown that direct uptake of RNA into eukaryotic cells is also possible. dsRNAs injected into the nematode (Fire et al., 1998) were able to cause phenotypic effects which were not limited to injection into specific cells, and thus must have required uptake from the extra-cellular fluid. Perhaps more

surprisingly, feeding dsRNAs produced in situ by engineered bacteria (Timmons and Fire, 1998), or even soaking nematodes in dsRNA solution (Tabara et al., 1998), could also produce changes in phenotype as a result of RNAi activity. Both the feeding and soaking methods also depend on uptake of dsRNA into cells, either from the gut, or from the surface of the nematode. In addition to import of RNA into cells through the uptake mechanism, RNA must also be exported from cells, since RNAi effects in *C. elegans* show a systemic effect. Specific injection of one tissue with dsRNA can lead to phenotypic effects of RNAi being observed in other tissues, or in progeny from the injected nematode (Fire et al., 1998).

The mechanism(s) by which dsRNA is able to cross the cell membrane in nematodes has yet to be fully described. Three genes, *sid-1*, *-2* and *-3*, were identified in a genetic screen as necessary for systemic RNA effects (Winston et al., 2002), and the product of *sid-1* (SID-1) was characterised as a trans-membrane protein located in the cell plasma membrane, which was hypothesised to act as a channel for dsRNAs and/or siRNAs, or possibly as a receptor mediating their endocytosis. SID-1 was absent in *C. elegans* neuronal tissue, which is refractory to RNAi effects caused by exogenous dsRNA. *D. melanogaster*, which does not contain a homologue of *sid-1*, does not show systemic RNAi effects, but expression of SID-1 in *Drosophila* cells enabled passive uptake of dsRNA (Feinberg and Hunter, 2003; Shih et al., 2009). SID-2 in *C. elegans* was later characterised as a trans-membrane protein, localised in the intestine, and required for import of dsRNA from the gut lumen of the nematode into the body cavity. Transport was considered to occur via a transcytosis mechanism. Both *sid-1* and *sid-2* are necessary for RNAi effects via feeding (Fig. 8.2). SID-1 cannot mediate uptake from the intestinal lumen, but only *sid-1* is required for RNAi effects via injection into the body cavity, since only SID-1 is used to transport dsRNA into the cell cytoplasm (Winston et al., 2007).

Unfortunately, this simple picture is complicated by a number of factors. Other genes which may play a role in RNA transport have been identified in *C. elegans* (Tijsterman et al., 2004). A functional genomic analysis to identify genes necessary for RNAi effects in *C. elegans* (Kim et al., 2005) failed to identify any of the *sid* genes, but did identify *rsd-2*, a gene previously identified as required for spread of systemic RNAi effects (Tijsterman et al., 2004). Five *rsd* genes were identified in the earlier screen, including: *rsd-2* and *rsd-6*, which were shown to encode proteins which formed a complex, mediating systemic RNAi; *rsd-3,* which encoded a protein involved in vesicle trafficking, and *rsd-8*, which was shown to be identical to *sid-1*. In addition, *rsd-4* may be identical to *sid-2* (Jose and Hunter, 2007). Some of these genes may be involved in RNA exocytosis. Exocytosis was first shown in insect cells; wild-type *Drosophila* cells were able to take up dsRNA from exogenous media via a slow, energy-dependent mechanism, mediated by pattern-recognition receptors, which was characterised as exocytosis (Saleh et al., 2006; Ulvila et al., 2006). A similar mechanism is present in *C. elegans*, since suppression of expression of genes encoding components of the endocytotic pathway by RNAi results in worms with a "loss-of RNAi function" phenotype (Saleh et al., 2006). A recent paper (Jose et al., 2009) has concluded that import of dsRNA into cells is dependent

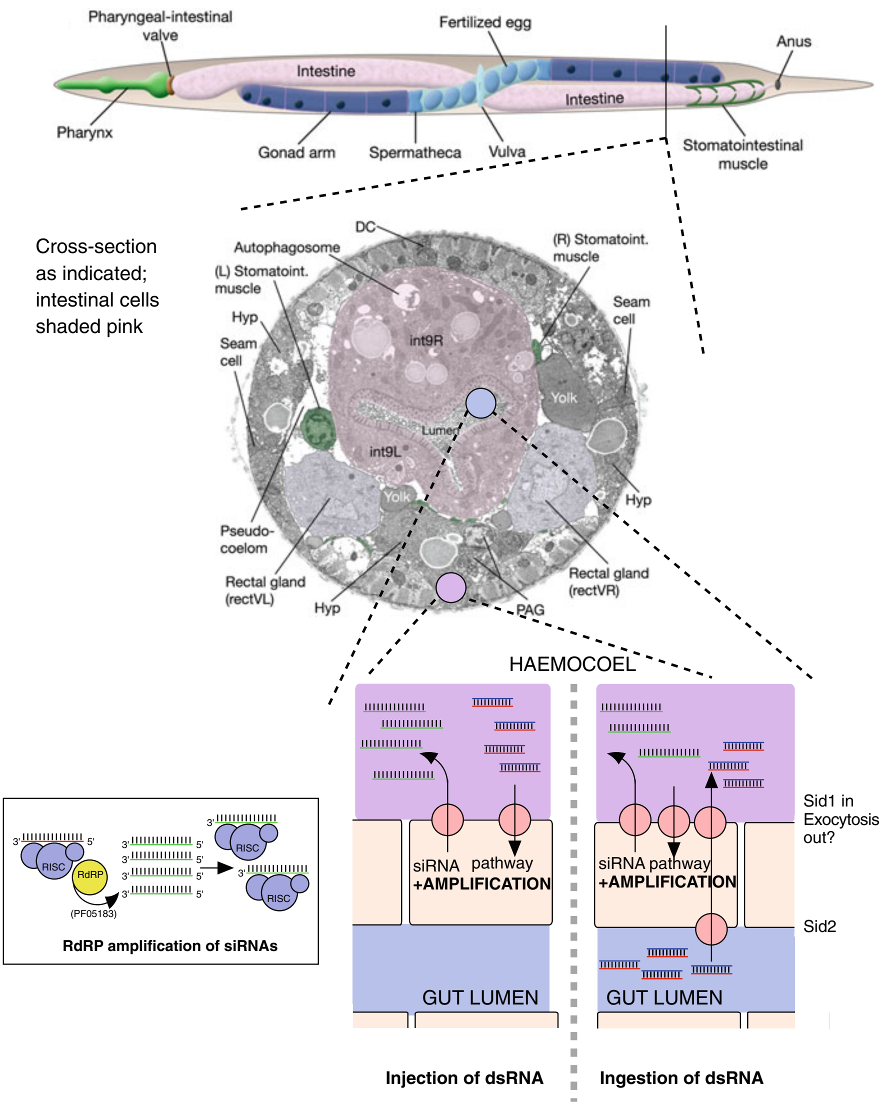


Fig. 8.2 Import and export of RNAi in the nematode *C. elegans*. The transporter SID-1 is necessary for import of dsRNA into cells, but both SID-1 and SID-2 are necessary for RNAi effects as a result of ingesting dsRNA. SID-1 alone can mediate RNAi effects from injected dsRNA, and is thought to be the major import route for dsRNA into cells. SID-2 may be involved in a transcytotic mechanism which transports dsRNA directly across intestinal cells to the body cavity; presumably intestinal cells do not express SID-1 on the surface facing the gut lumen. Uptake of dsRNA may also occur through sensory pores in the collagen surface of the nematode. Export of RNAi effects from one cell to another (systemic RNAi) depends on amplification of the original siRNAs by RNA-directed RNA polymerase (RdRP) and a further transport mechanism (possibly involving products of *rsd* genes and exocytosis). *C. elegans* diagram and section based on Altun and Hall (2008), with thanks

on SID-1, but that export of the RNAi triggers which mediate the systemic effect is not dependent on SID-1, and thus multiple pathways for RNA import and export are present.

Transport of dsRNA (and other possible "RNAi triggers" – see below) into and out of cells is an important factor in determining whether RNAi is a practical method for crop protection against insects. Although the basic intra-cellular mechanisms which lead to RNAi effects are present in all eukaryotes, the cellular import and export mechanisms for RNA differ between different phyla, and sometimes between different orders. Plants can readily transmit RNAi effects over long distances via the phloem (Kehr and Buhtz, 2008), but this is not the case in many animals, and the efficiency of uptake of dsRNA from the extra-cellular medium differs greatly among animals; thus although *Drosophila* cells can take up exogenous dsRNA to produce an RNAi effect, the effect is greatly increased if the cells express *C. elegans* SID-1. In the absence of a systemic RNAi effect, only cells directly exposed to dsRNA will show a phenotype as a result of siRNA, and even then the phenotype may only be produced at low efficiency due to poor import of dsRNA. Whereas *Drosophila* cells are able to take up dsRNA when in culture, extra-cellular injection of dsRNA in most *Drosophila* tissues does not produce an RNAi effect, although haemocytes are able to take up exogenous dsRNA effectively (Miller et al., 2008).

Many experiments have used injection into the haemocoel or abdominal cavity (insects), or into the circulatory system (mammals) to attempt to demonstrate systemic RNAi effects, often with considerable success in showing that effects can be produced in different tissues (see Jose and Hunter, 2007). However, these experiments do not usually establish that export of RNA from cells exposed to the injected RNA signal occurs, since the animal's circulatory system effectively distributes RNA, and the observation of effects in distant tissues from the injection site only requires import of nucleic acid from circulatory fluid. In a true systemic RNAi effect, RNAs are exported from cells which are exposed to the original RNA signal, and produce effects in other cells. The export process is preceded by the synthesis of new RNAs ("RNAi triggers"), which leads to an amplification of the original RNAi effect, and its persistence with time in the organism, leading to transmission to progeny. This amplification of the original RNA signal has been demonstrated in *C. elegans* (although the nature of the transmitted "RNA trigger" has not been fully characterised), but its presence or absence in other animals remains controversial (Jose and Hunter, 2007). Evidence suggests that it is absent in the model insect species, *D. melanogaster* (Roignant et al., 2003), since RNAi effects produced by expressing hairpin dsRNAs endogenously within cells are not transmitted to other cells. Although spread of dsRNA, and RNAi effects in distal cells, dependent on the exocytotic uptake mechanism, has been observed during viral infections in *Drosophila* (Saleh et al., 2009) this does not show endogenous amplification of the RNAi, since the systemic effect in this case is based on viral generation of dsRNA fragments.

Systemic RNAi effects in animals are normally considered to depend on RNA-dependent RNA polymerase (RdRP). In *C. elegans*, this enzyme interacts with the RISC complex, and produces secondary siRNAs using the bound siRNA as a template; it is the enzyme necessary for the RNA amplification step leading to

persistent and systemic RNAi effects (Sijen et al., 2001). A domain, designated PF05183 in the PFAM database, is characteristic of RdRP, and has been identified in gene products of eukaryotic microorganisms, fungi, plants, nematodes and a primitive vertebrate (*Branchiostoma floridae* – a cephalochordate), but not in insects, molluscs, or other vertebrates. The absence of RdRP in insects would suggest that an RNA amplification/export system is not present, consistent with the failure to observe systemic RNAi effects from endogenously produced dsRNA in *Drosophila*.

Limitations on the delivery of RNA to herbivorous insects make production of a systemic RNAi effect desirable if the technique is to be effective. If an RNA amplification/export system were present in insects, genes expressed anywhere in the insect, at any stage of the life cycle, could be selected for down-regulation by specific dsRNA delivered by a plant host, rather than just genes expressed in gut tissue during the feeding stages. In addition, the RNAi amplification step would avoid the necessity of supplying high levels of dsRNA continuously, and thus could avoid many of the problems associated with instability of dsRNA in the insect gut. Fortunately, RNAi effects in *Drosophila* do not reflect the situation for all insects. The red flour beetle, *Tribolium confusum*, shows a robust systemic RNAi response, which can be transmitted to progeny. The systemic response was demonstrated using a homologue of the *Drosophila* sensory bristle-forming gene *Tc-achaete-scute* (*Tc-ASH*); larvae injected with Tc-ASH dsRNA at a single site showed a "loss-of-bristle" phenotype over the entire epidermis of adult insects (Tomoyasu and Denell, 2004). The parental RNAi effect transmissible between generations was shown using genes regulating development; injection of dsRNA corresponding to (1) *Distalless* (leg development gene), (2) *maxillopedia* (homeotic gene) and (3) *proboscipedia* (encoding a homeotic protein required for the formation of labial and maxillary palps) caused phenotypic changes characteristic of an RNAi effect in both injected insects and progeny embryos (Bucher et al., 2002). Virtually all *T. confusum* tissues respond strongly to extra-cellular dsRNA (Miller et al., 2008), suggesting that RNA import into cells is much more efficient in this organism than in *Drosophila*. Although direct evidence for RNA amplification has not been observed in *Tribolium*, amplification by a mechanism distinct from that observed in *C. elegans* has not been ruled out either, especially in view of a genome-wide survey which concluded that many components of the RNAi response differed between nematode (*C. elegans*) and beetle (*T. confusum*) (Tomoyasu et al., 2008). The presence of three homologues of *sid-1* in *T. confusum*, and the absence of a *sid-1* homologue in *Drosophila* might be considered causative in determining the presence or absence of a systemic RNAi effect in the two insects, but analysis of other insect genomes does not produce a clear correlation between the presence of *sid-1* homologues and systemic RNAi responses, and the conclusion was drawn that transport mechanisms for dsRNA import differ between nematodes and insects (Tomoyasu et al., 2008). Cellular RNA export mechanisms for systemic RNAi effects in insects are not characterised at present. Other insects besides *Tribolium* (e.g. the wasp *Nasonia vitripennis*; Lynch and Desplan, 2006) are capable of transmission of RNAi effects from parents to progeny, and thus RNA export must occur in these species also.

The present state of knowledge shows that RNAi effects can be produced in a wide range of insects; specific suppression of gene expression has been reported in Diptera, Coleoptera, Lepidoptera, Hymenoptera, Orthopetra, Blattodea, and Hemiptera [Misquitta and Paterson, 1999; Bucher et al., 2002; Rajagopal et al., 2002; Amdam et al., 2003; Dong and Friedrich, 2005; Cruz et al., 2006; Mutti et al., 2006], usually by appropriate injection of dsRNA. However, the efficiency of the process is variable between different species, as is the presence of any systemic effects. Long dsRNAs are used to cause RNAi effects in insects, in contrast to mammals where short siRNA-sized fragments are necessary to avoid causing an immune response. siRNAs are not taken up efficiently by *Drosophila* cells in culture, whereas dsRNA is taken up more readily (Saleh et al., 2006). Both short and long dsRNAs are effective in inducing RNAi effects when injected into *C. elegans*, but only longer dsRNAs (>100 bp) induce systemic effects (Feinberg and Hunter, 2003); the SID-1 transporter does not import short or long dsRNAs at different rates on a weight basis (Shih et al., 2009) and thus the differences in effects must be due to part of the RNA amplification/export process. Whether similar considerations apply in insects is not yet known.

8.4 Oral Delivery of dsRNA to Insects to Produce RNA Interference Effects

The simple methods available for producing RNAi effects in *C. elegans*, where dsRNA can be fed or applied exogenously, and is transported into cells, show a high degree of species-specificity. A closely related nematode, *C. briggsae*, does not show RNAi effects when soaked in dsRNA solutions (Winston et al., 2007), and this lack of an environmental RNAi effect was used as a functional assay for the *C. elegans sid-2* gene. With this result in mind, it is not surprising that attempts to produce RNAi effects in insects using delivery methods for dsRNA other than injection have been met with highly variable degrees of success; as discussed above, both import of dsRNA and the RNA amplification/export process vary in their effectiveness between different species, and there are wide variations in gut physiological conditions and biochemistry which may affect RNA stability, as well as transport processes.

Evidence that dsRNA fed to an arthropod, rather than injected, could provide an RNAi effect was first observed in the tick *Ixodes scapularis* (Soares et al., 2005), where specific suppression of expression of an anti-complement gene (*isac*) was produced by feeding dsRNA solution to nymphal ticks by capillary. This species is a blood feeder, and thus delivery of RNA in solution was readily achievable. Suppression of gene expression was demonstrated by northern blot, and a phenotypic effect on growth was observed. Subsequently, a similar experiment was carried out with a true insect, the bug *Rhodnius prolixus* (Hemiptera). This species is a blood feeder like the tick *I. scapularis*, and, as for the arthropod, an RNAi response was observed on feeding dsRNA in solution. A salivary gland transcript, nitroporin 2 (NP2) was specifically down-regulated by dsRNA both when fed and by

injection into the thorax (Araujo et al., 2006). Injection was more effective in suppressing gene expression (75% reduction) than feeding dsRNA (42% reduction). Gene suppression by RNA feeding has also been shown to be a usable technique in bee (*Apis mellifera*) larvae (Aronstein et al., 2006; Patel et al., 2007; Franco Nunes and Paulino Simoes, 2009) and termite (*Reticulitermes flavipes*) juveniles (Zhou et al., 2008). Whereas dsRNA fed to bee larvae was incorporated into a semi-liquid diet, dsRNA fed to termites was dried down on filter paper, illustrating that RNA stability prior to ingestion is not a serious drawback to this delivery method. A significant indication of the limitations of suppression of expression of specific genes by feeding dsRNA was observed in a study of RNAi effects in the tsetse fly (*Glossinia morsitans morsitans*), another blood feeder (Walshe et al., 2009). Delivery of dsRNA in the bloodmeal was shown to be effective in suppressing expression of an immunoresponsive midgut-expressed gene, *TsetseEP*, with inhibition of expression (up to 50%) comparable to injection of dsRNA. However, feeding dsRNA was ineffective in suppressing expression of a transferrin gene expressed in the fat body. These results show that midgut cells exposed to dsRNA can take it up, and produce an RNAi effect endogenously, but that there is no systemic effect. Presumably, dsRNA is not transported across the gut in this insect, and is not exported from midgut cells to distal tissues (Fig. 8.3).

The applicability of suppression of gene expression by specific dsRNAs was also extended to herbivorous insects. Herbivorous lepidopteran larvae (light brown apple moth; *Epiphyas postvittana*) were fed dsRNAs in solution by droplet feeding (Turner et al., 2006). Specific suppression of expression of a gut carboxylesterase gene (*EposCXE1*) was observed after 2 days of feeding, with up to 80% decrease in expression relative to controls after 7 days. *EposCXE1* is expressed in gut tissue, and thus only local dsRNA uptake was required for the RNAi effect to be observed. However, feeding a specific dsRNA to larvae also suppressed expression of *EposPBP1*, a gene encoding a pheromone-binding protein expressed in the adult antenna, with approximately 60% suppression relative to controls persisting 2 days after emergence from pupae. This result demonstrated a persistence of the RNAi signal throughout larval-adult stages (implying RNA amplification) and a systemic RNAi effect involving transport from gut tissue to the antennae. This insect is comparable to *Tribolium* in showing a strong systemic RNAi effect, while in addition it is able to take up dsRNA from the gut effectively. In contrast, an earlier report had shown that an aminopeptidase-N gene expressed in the midgut of larvae of a different lepidopteran herbivore (*Spodoptera litura*) could not be down-regulated by feeding dsRNA, although injection of the same RNA into the insect haemocoel gave a strong RNAi response (Rajagopal et al., 2002). Similarly, silkworm (*Bombyx mori*) larvae do not show a robust systemic RNAi response (Tomoyasu et al., 2008). However, feeding chemically synthesised siRNA to larvae of corn earworm (*Helicoverpa armigera*) in diet did result in specific suppression of expression of an acetylcholinesterase gene (Kumar et al., 2009), with effects observed at mRNA and protein levels, and feeding dsRNA to larvae of diamondback moth (*Plutella xylostella*) also produced RNAi-mediated gene suppression (Bautista et al., 2009). The results with *E. postvittana* were achieved with doses of dsRNA as small as 4 μg,

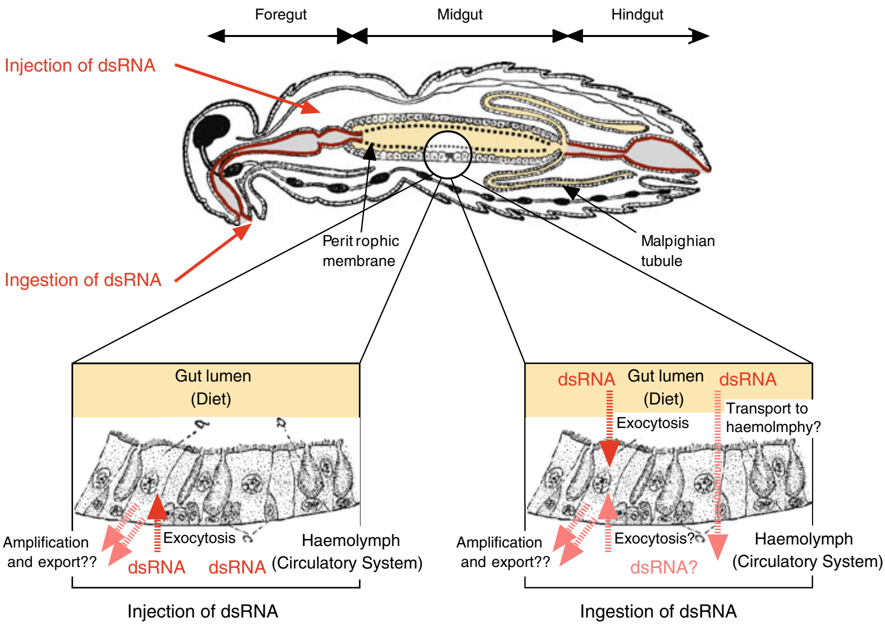


Fig. 8.3 RNAi effects in insects from injection and ingestion of dsRNA. dsRNA injected into the body cavity causes RNAi effects throughout the insect as a result of import into cells, probably via a mechanism mediated by exocytosis, although proteins of similar function to *C. elegans* SID-1 may also be present. The efficiency of uptake varies greatly between cell types, and between insect species. Systemic RNAi effects also vary greatly between insect species, and mechanisms of siRNA amplification and export (if present) remain uncharacterised. Ingestion of dsRNA can deliver RNAi effects to midgut cells and Malpighian tubule cells in contact with the gut lumen, possibly via transport into these cells by an exocytotic mechanism. Delivery to other cells is prevented by the chitin lining (brown) of foregut and hindgut, although pores in this lining may allow access to cells with sensory and transport functions. It is not known whether dsRNA is transported into the circulatory system to produce any systemic effects observed, or whether siRNA amplification and export takes place

at a concentration of 1 μg/μl; these figures are significant in that they are feasible to achieve in a plant-based delivery system. The work of Turner et al. (2006) therefore provided a basis for subsequent strategies in which plants were engineered to produce dsRNAs designed to give protection against insect pests (see below).

Further feeding studies with insect herbivores have been carried out with pea aphid (*Acyrthosiphon pisum*), where oral administration of dsRNA at 1 μg/μl in liquid diet resulted in a two-fold suppression of expression of the aquaporin gene *ApAQP1* at 1 day after exposure, which was transient, decreasing to no suppression after 7 days (Shakesby et al., 2009). The result suggests that there is no amplification-based systemic RNAi effect in this insect, since the gene suppression is not persistent. Similar results had been obtained previously by injection of RNAi into the aphid body cavity, where transient suppression of expression of calreticulin (*Ap-crt*) and cathepsin (*Ap-cathL*) genes was observed (Jaubert-Possamai

et al., 2007). In contrast, the lethal effects produced by injection of dsRNA directed against a salivary gland transcript (Mutti et al., 2006) involved large decreases in the level of the corresponding transcript. Aphids contain homologues of the *C. elegans sid-1* gene (Xu and Han, 2008), but as discussed above, the presence or absence of sid-1 homologues in insects is not a predictor of robust systemic RNAi effects (Tomoyasu et al., 2008). In contrast, feeding dsRNA specific for a gene encoding arginine kinase to the beetle *Phyllotreta striolata* (Zhao et al., 2008) caused mortality, impaired development, reduced fecundity and reduced fertility, suggesting a much stronger RNAi effect than observed in aphids.

In conclusion, specific gene suppression through RNAi effects by feeding dsRNA is possible in insects, but the efficacy of the technique varies widely from species to species, and criteria for predicting its success or failure in particular cases have yet to be formulated. A more systematic approach to examining the factors responsible for determining the success of the technique, namely stability of the input RNA in the insect diet and in the insect gut, transport of RNA across the insect gut, and uptake into insect cells in vivo will lead to a better understanding of how to maximise the effects that can be produced.

8.5 Production of dsRNA in Plants for Delivery to Invertebrate Pests: Nematodes as a Case Study

Having established that dsRNA fed to insects can cause suppression of expression of specific genes, the next step in developing a practical crop protection method is to produce the dsRNA in the plant material which is ingested by insect herbivores. The technology necessary to produce dsRNA in plant cells was developed as a method for producing resistance to plant viruses, most of which contain a single-stranded RNA genome, which replicates via a transient dsRNA. The dsRNA triggers siRNA formation in the plant, leading to degradation of the viral genome as a natural defence response (reviewed by Tenllado et al., 2004). The virus normally produces one or more factors to suppress the formation of active RISC complexes, to allow replication to occur. If plants are "primed" by preintroduction of dsRNAs corresponding to genes in the viral genome necessary for replication, then resistance to the virus results, since the virus is unable to produce factors which suppress the plant's defence against it. Exogenous dsRNAs can be introduced into plant cells by mechanical inoculation or injection (or even external application; see Tenllado et al., 2004) or by transient expression of suitable constructs from extracellular *Agrobacterium tumefaciens* bacteria (Johansen and Carrington, 2001), but stable resistance is best achieved by transformation of the plant genome with a suitable construct that will lead to the production of dsRNA (Waterhouse et al., 1998; Smith et al., 2000). Transgene constructs which contain an inverted repeat, so that a hairpin RNA structure is formed, in which the double-stranded region corresponds to coding sequence, can lead to almost complete suppression of expression of endogenous plant genes (Smith et al., 2000), and give effective protection against

viruses when a suitable viral gene is targeted by the dsRNA region (Kalantidis et al., 2002). The single-stranded loop can correspond to an intron in the targeted gene, but need not do so.

The suppression of genes in pathogens by plant-produced dsRNA has been extended to give resistance to bacteria (Escobar et al., 2001), and more significantly, to plant parasitic nematodes (reviewed by Bakhetia et al., 2005; Gheysen and Vanholme, 2007; Lilley et al., 2007). The observation that species of nematodes which attack plants were also susceptible to RNAi effects mediated by oral ingestion of dsRNA (Urwin et al., 2002), or by soaking eggs (Fanelli et al., 2005) or juvenile nematodes (Kimber et al., 2007) in dsRNA solution as is the case for *C. elegans*, makes delivery of dsRNA by the plant host viable. Introgression of molecules from surface pores or sense organs, and through the nematode feeding structure, have been shown to be subject to size limitations, which limit the size of proteins that can be taken up to <20–50 kDa (depending on species), but dsRNA may be able to avoid this limitation, possibly due to the linear nature of the molecule (discussed in Lilley et al., 2007). However, doubts have been cast on the ability of cyst nematodes, such as *Heterodera schachtii* and *Globodera pallida* to ingest sufficient dsRNAs to produce strong RNAi effects, due to a lower size exclusion limit in the feeding structure (Gheysen and Vanholme, 2007). Establishment of the feeding site involves exposure of the nematode to molecules present in the extra-cellular spaces in plant tissue, molecules secreted from the roots, and molecules present in cell contents leaking from damaged cells, while feeding by the nematode involves direct ingestion of cell contents. Uptake of dsRNA from the gut after induced ingestion from soaking solution is a reproducible technique for producing RNAi effects in plant parasitic nematodes, and thus delivery via cell contents is the method of choice for plant-expressed RNAs, and is technically the most simple to achieve. Like *C. elegans*, plant parasitic nematodes show a systemic RNAi effect, with suppression of gene expression in tissues distant from the gut (such as gonads) being observed after feeding, although the evidence for RNA amplification and export is not conclusive, since persistence of RNAi effects is only partial, and transmission of effects to progeny has not been fully characterised.

Engineered plants expressing dsRNAs directed against nematode genes are now a well-established resource. Yadav et al. (2006) described transgenic tobacco plants expressing hairpin dsRNAs corresponding to sections of coding sequences of genes encoding a splicing factor and integrase (a chromatin remodelling protein) from a root knot nematode (*Meloidogyne incognita*). Nematodes exposed to these plants showed specific suppression of expression of the target genes, and the plants were strongly resistant to nematode attack, as judged by successful development of nematodes to reproductive maturity. In a similar but less complete study, expression of a dsRNA corresponding to a gene encoding a nematode secretory peptide (16D10) was engineered in *Arabidopsis*. Although suppression of gene expression in nematodes was not directly demonstrated, the dsRNA-expressing plants gave reductions in egg production from mature females of 63–90% in four related root knot nematode species exposed to plants as juveniles (Huang et al., 2006). A root knot

nematode transcription factor gene (*Meloidogyne javanica MjTis11*) was targeted by dsRNA in engineered tobacco plants, and consistent evidence for silencing of the gene in nematodes feeding on transgenic plant roots was obtained, although no phenotypic effects were observed (Fairbairn et al., 2007). This study showed that plants engineered to express dsRNAs contained siRNAs derived from the dsRNA, although it is not known whether these are also effective in causing an RNAi effect in the nematode after ingestion. Plant delivery of dsRNA to cause an RNAi effect in cyst nematodes was also reported; transgenic soya beans expressing dsRNA from a construct containing an inverted repeat of a cDNA clone of a major sperm protein of *Heterodera glycine* (soybean cyst nematode) were produced, and were shown to contain siRNAs corresponding to the introduced gene construct. Direct evidence for suppression of gene expression was not obtained, but reproduction in nematodes exposed to the plants was inhibited, as shown by a 70% reduction in egg production. Surprisingly, this reduction in reproductive capacity was also observed in progeny from the initial generation of nematodes exposed to transgenic plants, implying a systemic, persistent RNAi effect. Subsequent work has targeted different nematode genes for RNAi-mediated suppression of expression; expression of dsRNAs corresponding to genes encoding small ribosomal proteins, synaptobrevin and a spliceosome component in transgenic soya bean roots all caused at least 80% inhibition of nematode development (Klink et al., 2009), although again direct evidence for RNAi effects on gene expression were not shown. Four genes involved in parasitism were targeted in the cyst nematode *Heterodera schachtii* (sugar beet cyst nematode) by expression of dsRNA constructs in *Arabidopsis*. Transgenic plants contained dsRNA fragments and siRNAs, and nematodes feeding on the plants showed specific down-regulation of expression of the targeted genes, with decreases in mRNA levels of up to twofold. The resulting effect on nematode development was limited (up to 60% reduction in females), but the validity of the RNAi effect was demonstrated (Sindhu et al., 2009).

The continuing development of nematode-resistant plants using expression of dsRNAs directed against nematode genes has shown that the basic strategy is viable, and that feeding from plant cell contents can produce an RNAi effect in an invertebrate herbivore. Expression of dsRNAs from a strong constitutive promoter (CaMV 35S) does not lead to phenotypic abnormalities in transformed plants (Sindhu et al., 2009), and the presence of siRNAs and dsRNA fragments in extracts shows that dsRNA is present in cells. There is thus good reason to suppose that similar techniques could be used to produce plants expressing dsRNAs directed against genes in insect herbivores. However, two potential drawbacks to this strategy are apparent. First, the endogenous siRNA production system in plants is clearly capable of rapidly converting dsRNAs produced as hairpin constructs, or assembled in vivo from separate strands, into small single-stranded RNAs. These small RNAs are less stable than dsRNAs, and are less effective in inducing RNAi effects in insect cells, due to poor uptake (Saleh et al., 2006). Second, to target one major class of insect pests, hemipteran sap-sucking species, dsRNA (or other inducers of an RNAi response) should be present in phloem sap, not inside cells, since these insects cause minimal tissue damage when feeding. These insects are not amenable to control

by current transgenic plant technology, based on expression of genes encoding *B. thuringiensis* toxins (Gatehouse, 2008), and are thus major targets for alternative control strategies, such as RNAi-based effects. Targeting of RNA to phloem sap in plants is not an unrealistic goal, since long-distance transport of RNA in phloem has recently become well established as part of the plant's signalling repertoire (reviewed by Kehr and Buhtz, 2008). Viral RNA is known to transport readily through phloem sap, the mobile form being a complex of single-stranded RNA with a virally encoded movement protein, which enables the complex to pass through plasmodesmata (Citovsky et al., 1990). However, some cellular mRNAs are also present in phloem (Sasaki et al., 1998), and can spread through the phloem network. More relevantly, plant siRNAs also are able to transport via the phloem (Yoo et al., 2004). Evidence suggests that siRNAs transport in the phloem as single strands, and are possibly associated with protein chaperones. An export mechanism to introduce siRNAs into the phloem is present, and cells can take up siRNA strands from the phloem, allowing post-transcriptional gene silencing to be propagated from one part of the plant to another. The potential utility of the endogenous plant siRNA phloem transport system in generating RNAi effects in sap-sucking pests will depend on whether these insects will take up siRNA to generate an RNAi effect.

8.6 Insect Resistance in Plants Through RNAi Effects: Current Progress

To produce plants that are protected against insects through expression of dsRNA targeted against pest genes, two conditions must be satisfied: (1) enough dsRNA must be produced in the plant and enough "RNAi trigger" (usually dsRNA fragments, but possibly siRNA – see above) delivered to the pest to produce an RNAi effect; (2) the RNAi effect must cause a sufficient change to the insect phenotype to give resistance (optimally, to cause mortality, but other effects such as preventing feeding, or blocking development and/or reproduction could be sufficient in certain cases). Work aimed at producing nematode resistance in plants, described above, has made clear that condition (1) can be satisfied, at least for insects which consume plant tissue (i.e. ingest cell contents). However, the nematode case study has suggested that condition (2) is more difficult to satisfy; the choice of gene target for down-regulation is crucial, both in terms of how strong the RNAi effect is (i.e. by what factor gene expression is reduced) and in the choice of gene for down-regulation (i.e. what phenotypic effect results from suppression of gene expression). Effective suppression of a gene that results in no phenotype change, as was the case for the nematode transcription factor gene *MjTis11* (Fairbairn et al., 2007) is of academic rather than practical interest.

The first two papers to show that transgenic plants expressing dsRNAs directed against insect genes showed enhanced resistance used different, complementary strategies to identify target genes for RNAi.

8.6.1 Preselection of Target Gene

The strategy followed by Mao et al. (2007) involved preselection of a gene in the pest, which would produce a deleterious phenotype when its expression was suppressed by the RNAi effect. The example chosen involved an insect gene whose product was necessary for detoxification of a secondary metabolite in the plant host. Cotton bollworm, *H. armigera*, is a lepidopteran pest which is able to detoxify the major endogenous defence compound in cotton, the sesquiterpene aldehyde gossypol. The gene *CYP6AE14* was identified as specifically up-regulated over 100-fold in midgut tissue when larvae were transferred to diets containing the compound, and was linked to gossypol tolerance by expression pattern, localisation of protein accumulation, and correlation of accumulation with larval growth on gossypol-containing diets. The enzyme was concluded to be responsible for gossypol metabolism, leading to detoxification. A construct containing an inverted repeat derived from the *CYP6AE14* coding sequence was produced and introduced into tobacco and *Arabidopsis*; accumulation of dsRNA in *Arabidopsis* leaves was demonstrated by northern blotting. Larvae fed on transgenic material producing the dsRNA contained siRNAs derived from it in midgut tissue, and showed suppression of expression of *CYP6AE14*, to the limit of detection on northern blots in the best case. CYP6AE14 protein accumulation was decreased, and the larvae showed slightly reduced growth (possibly due to reduction in capacity to detoxify secondary metabolites in tobacco and *Arabidopsis* leaves). Transfer to diets with and without gossypol, or cotton leaves containing gossypol, showed that the larvae had lost the capacity to detoxify the compound, and growth was completely arrested. The RNAi strategy has thus prevented the insect from responding to a naturally occurring plant defensive compound, and restored its effectiveness towards this particular pest. The suppression of expression of another midgut-expressed gene in bollworm, a glutathione transferase, by a specific plant-expressed dsRNA was also reported, showing the general applicability of the technique. Control dsRNA had no effect.

The convincing demonstration of RNAi effects by plant-expressed dsRNA in this paper relies only on RNA uptake into gut cells in the insect pest; the work of Turner et al. (2006) had previously shown that lepidopteran larvae produce a robust RNAi response in the gut when fed dsRNA. Whether systemic effects could be produced by this plant delivery method remains to be determined. The paper contains some interesting indications that processing dsRNA to siRNA may limit the effectiveness of plant-delivered RNAi effects. An *Arabidopsis* mutant with three of its four Dicer-like (*dcl*) genes inactivated accumulated significantly more dsRNA in leaves than wild-type *Arabidopsis* when transformed with the *CYP6AE14* dsRNA construct, and had reduced levels of corresponding siRNAs; silencing effects on *CYP6AE14* gene expression in midguts of larvae feeding on leaves from mutant plants were greater than observed with leaves from wild-type plants with lower levels of intact dsRNA. This observation suggests that dsRNA is more effective than siRNA in inducing an RNAi effect in the insect, as previously deduced from import studies using cultured insect cells (Saleh et al., 2006). The evidence points to a basic incompatibility between the plant's endogenous systems, which process and degrade dsRNA, and

transport RNA in a single-stranded form (see above), and a requirement to deliver dsRNA to the insect to produce an effective RNAi effect. However, the results show that this need not prevent the technique from working to a level required to give acceptable protection.

8.6.2 Selection of Target Genes by Screening

A more "industrial" approach was taken in the paper by Baum et al. (2007a), where the target pest was a coleopteran species, western corn rootworm (*Diabrotica virgifera virgifera*). Clones were selected from *D. virgifera* cDNA libraries on the basis of the encoded polypeptide being predicted to provide "essential functions", and were used to produce dsRNA in vitro. The dsRNA was fed to *D. virgifera* larvae by surface application at defined concentrations (ng cm^{-2}) on artificial diet, and subsequent larval mortality was assayed. An initial "cut-off" screen was carried out on 290 dsRNAs, and the assays were repeated with active RNAs at different doses to produce dose–response curves. The screen enabled 14 dsRNAs which showed high insecticidal activity at low concentration to be identified. Northern blots carried out on insects fed dsRNAs showed strong suppression of expression of the corresponding genes. In the case of a gut-expressed gene (V-type ATPase, subunit A; *V-ATPase A*) mRNA in the insect was virtually absent after 1 day exposure to dsRNA, whereas in the case of a "housekeeping" gene expressed throughout the insect (*α-tubulin*) suppression of expression occurred at a slower rate, but was virtually complete after 6 days. This result suggests that a systemic RNAi effect was occurring in the insect, since tubulin genes are expressed in tissues distant from the gut, and not accessible to gut contents. On the basis of the low levels of dsRNA necessary for toxicity, and the slow onset of mortality, the authors suggested that RNA amplification and export occurred in *D. virgifera*. dsRNAs based on the entire coding sequence of a target gene, *V-ATPase A* (1,842 bp) and 300-bp fragments of the coding sequence were equally effective in producing an RNAi effect. A 246-bp section of the coding sequence was used to prepare a construct for expressing dsRNA as a hairpin structure in plants, with a strong constitutive promoter. Maize transformed with this construct contained both intact dsRNA, and 21-base siRNAs derived from it. When plants were exposed to larvae of *D. virgifera*, protection against damage to roots was observed; the degree of protection correlated with the level of expression of the dsRNA. The best transformants were protected to a level that would be considered commercially viable.

This paper contains some important data on potential specificity of RNAi effects in insects feeding on engineered plants, which is of concern for use of the technology in the field. Larvae of three related coleopteran plant pests, Southern corn rootworm (SCR; *Diabrotica undecimpunctata howardii*), Colorado potato beetle (CPB; *Leptinotarsa decemlineata)*, and cotton boll weevil (*Anthonomus grandis* Boheman) were fed dsRNAs specific for three target genes identified in WCR, *β-tubulin*, *V-ATPase A* and *V-ATPase E*. Feeding dsRNAs based on WCR sequences caused mortality in SCR and CPB larvae. Dose–response curves showed that

concentrations of dsRNA necessary to cause mortality in CPB were greater than tenfold higher, correlating with sequence identities between WCR and CPB of 83 and 79% for *V-ATPase A* and *V-ATPase E*, respectively. dsRNAs based on the sequences of CPB *V-ATPase A* and *V-ATPase E* showed increased effectiveness in feeding trials compared with the WCR orthologues. These results show that specificity of dsRNA-induced RNAi effects can be predicted based on sequence similarity between the target species and potential non-target species feeding on the same material. However, the species–species variation in RNAi effects induced by feeding dsRNA was shown in assays with cotton boll weevil larvae, in which no mortality or other deleterious effects were observed, whether dsRNAs derived from WCR gene sequences, or from orthologous boll weevil genes were fed.

The paper by Baum et al. (2007a) shows a robust systemic RNAi effect by feeding dsRNA in a coleopteran species, in line with the effects seen on injecting dsRNA into *Tribolium* (see above). The demonstration of protection against a major pest by expressing dsRNA in a transgenic crop species is a significant advance for the technology, which has been protected by patent applications (Baum et al., 2007b, 2008). However, the screening process for potential targets for RNAi effects in the pest contains mainly highly expressed, housekeeping genes, including those encoding various ribosomal proteins, and components of conserved systems such as proteasomes and transport. These are all likely to be strongly expressed in gut tissue, as is V-type ATPase, which has been suggested to be used by insects to generate pH gradients used for transport processes (Patrick et al., 2006). While it could be argued that the screening method employed to select dsRNAs has given positive results, the procedure has only considered a subset of potential targets, limited by the resources necessary to carry out large numbers of dsRNA feeding assays. In addition, targeting processes conserved in all eukaryotes for suppression of gene expression are likely to decrease the specificity of the RNAi effect, and raises the possibility of deleterious effects on higher animals.

8.7 Prospects for RNAi-Mediated Crop Protection

Reviews written shortly after publication of the papers described in Section 8.5 above were optimistic in anticipating widespread adoption of the new RNAi technology for insect control (Gordon and Waterhouse, 2007; Price and Gatehouse, 2008). However, to date no further papers which describe transgenic plants expressing dsRNAs directed against genes in herbivorous insects have appeared, and comparatively few papers have provided additional evidence for specific gene silencing by feeding dsRNAs to insects, despite convincing evidence for the validity of the phenomenon. Part of the problem may be the variability in the RNAi effect between insect species, noted above, and technical problems may also be involved.

An important factor in delaying the introduction of RNAi in crop protection against insect pests may be the success of existing transgenic technologies giving resistance to coleopteran and lepidopteran herbivores in crops. Baum et al. (2007a)

compared the protection against corn rootworm attack produced in maize plants produced by expression of dsRNAs to transgenic maize engineered to produce a modified Cry3Bb Bt toxin. The Bt-expressing maize gave significantly higher levels of protection, with virtually no damage observed. Although the Bt-expressing maize was a commercial product, and dsRNA-producing maize was research material, the data did not provide a compelling reason for replacement of Bt-maize in the short term. In addition, the trials of dsRNA-producing maize were carried out in growth chambers, and no data on whether protection would be effective in the field has been published. There is no impetus to replace Bt-expressing transgenic plants for pest control where an effective Bt toxin has been developed, although reports of resistance to Bt toxins being observed in field populations of insects exposed to transgenic plants (Tabashnik et al., 2008; Moar et al., 2008) may lead to alternative strategies for crop protection receiving more attention in the future.

The development of crop protection based on RNAi effects is likely to remain stalled until an example of high levels of resistance to a major pest which cannot be controlled by Bt toxins is published. Unfortunately, indications suggest that RNAi effects are not readily produced in hemipteran herbivores by feeding dsRNA, and dipterans may be equally difficult, if *Drosophila* (which is a herbivore) is used as an example. To maximise chances of success, more feeding bioassays need to be carried out with gut-expressed genes that can easily be targeted by dsRNA; in this respect, the screening approach of Baum et al. (2007a) to identify potential targets could be applied more widely, although preselection of one or more of the candidate genes identified in that screen might save much effort and be equally valid in a range of target insect species. Prediction of gene targets which will have a lethal phenotypic effect when their expression is suppressed in the insect, based on a knowledge of insect physiology and biochemistry, remains a method of choice for most investigators (e.g. Kumar et al., 2009).

Delivery of dsRNA from plant to insect via accumulation in plant cell contents, while clearly possible (since both coleopteran and lepidopteran herbivores chew plant tissues and ingest cell contents) is compromised by the endogenous dsRNA-siRNA processing system, and more work needs to be done on determining whether siRNAs can be effective in inducing an RNAi effect if fed to insects, and, if so, at what concentration compared to dsRNA. The chemically synthesised siRNAs shown to have a silencing effect when fed to *H. armigera* by Kumar et al. (2009) were double-stranded 21-bp fragments, and showed a gene suppression effect at a concentration of approximately 0.6 μg/ml (but not lower) when soaked into diet. This result shows that double-stranded pre-siRNA fragments are active, and indicates that as long as dsRNA is produced in large excess over RISC complex components, the action of Dicer enzymes *in planta* to produce double-stranded pre-siRNA fragments will not abolish a potential RNAi effect, since the strands of the siRNAs are not separated until they are incorporated into the RISC complexes. Nevertheless, the results of Mao et al. (2007) suggest that larger dsRNAs are better than siRNA-sized dsRNAs at suppressing gene expression in insects after ingestion. No direct evidence is available to determine whether single-stranded siRNAs can be effective in producing an RNAi effect when fed to insects; this would be highly desirable, since delivery of dsRNA via phloem (required for sap suckers) is probably

not possible. Whether the inefficient uptake of siRNA observed in *Drosophila* cells (Saleh et al., 2006) would be sufficient to produce an effect, or could be improved by complexation with carrier proteins or other molecules remains to be investigated. Research into methods for improving RNA uptake by insect cells could be of considerable value in improving the prospects for RNAi-based pest control.

RNAi effects mediated by feeding in insects are likely to receive increasing attention as a method for identifying phenotypes produced by specific genes (e.g. Araujo et al., 2009, Shakesby et al., 2009), but the prospect of engineered crop plants protected from attack by insect pests through RNAi effects being developed for commercial use looks more remote at present. Realising the potential of this technology will require more research at both fundamental and applied levels.

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Chapter 9
Insect Transgenesis and the Sterile Insect Technique

Marc F. Schetelig and Ernst A. Wimmer

Abstract The establishment of broadly applicable insect transgenesis systems will enable the analyses of gene function in diverse insect species. This will greatly increase our understanding of diverse aspects of biology so far not functionally addressable. Moreover, insect transgenesis will provide novel strategies for insect pest management and the means to impair transmission of pathogens by human disease vectors. Especially the Sterile Insect Technique (SIT) might be improved by the use of transgenic approaches. The SIT represents an effective and ecologically safe method for area-wide pest control that reduces the pest population by mass release of sterilized organisms, leading to infertile matings and in consequence to a decline of the pest population. Although the SIT is already successfully applied for some species, each of its steps – mass-rearing, sex-separation for male-only releases, sterilization, and marking for monitoring – can be improved biotechnologically to optimize the efficiency and to reduce the costs of ongoing programs or to transfer this effective technique to a wider range of species. However, this powerful transgenic technology must be applied with great care to avoid harm to our environment.

Keywords Transposable elements · Pest management · GMO safety

9.1 Introduction

More than 25 years ago, an efficient method was reported for the first time to integrate exogenous DNA into the genome of an insect so that it is stably transmitted to subsequent generations as a transgene (Rubin and Spradling, 1982). This process of insect genetic transformation was first established in the vinegar fly, *Drosophila*

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melanogaster, by using the transposable element *P* (Rubin and Spradling, 1982) and has been further improved and elaborated to become very successful in many diverse experimental settings (Ryder and Russell, 2003). Already at that time, hopes were raised that transgenic approaches could also be applied to other insect species in order to better understand the biology of certain agricultural pests or human disease vectors. In addition, from the very beginning, this technology was expected to enable the creation of transgenic insects for new and efficient strategies to control insect pest populations or to change the pest status of certain human disease vectors (O'Brochta and Atkinson, 1998). Genetic transformation has revolutionized research with *D. melanogaster* and has been instrumental for making this vinegar fly such an important model organism. However, despite great efforts, the *P* element proved to be not functional outside of the Drosophilids (Handler et al., 1993) because of host-specific co-factor requirements (Rio and Rubin, 1988). With the identification of alternative and more promiscuous transposable elements, it is now possible to apply powerful transgenic technologies to a broad range of insect species. In this chapter, we will briefly describe the requirements for establishing transgenic technologies in an insect species as well as discuss prospective applications in basic science and especially in applications of the SIT to control agricultural pest organisms.

9.2 Features of Insect Transformation Systems

To generate genetically modified, transgenic insects, first an in vivo method must be established to introduce gene constructs into the germ-line cells of the target organism. Secondly, a genetic vector system that enables the integration of the transgene into the genome of this organism – such as a retrovirus or a transposable element – must be identified. Thirdly, the transgene needs to be marked so that transgenic progeny can be easily recognized.

Establishing a transformation protocol. For the moment, this seems to be the most difficult part on the way to transgenesis of an insect species. Since the transformation rate for most species is still quite low, embryos should be readily available in large quantities. In addition, one should know where the primordial germ cells form in the embryo, so that the transgene vectors can be delivered to the appropriate place. In this respect the identification of a homologue to the conserved germ cell determinant VASA (Raz, 2000) will be helpful to identify where the germ plasm resides or primordial germ cells form during early developmental stages (Ewen-Campen et al., 2010; Extavour and Akam, 2003).

Electroporation (Swartz et al., 2001), biolistics (Thomas et al., 2001), sonoporation and lipofection (Lee et al., 2005) are potential ways of delivering transgene constructs to respective tissues. However, only biolistics has recently been demonstrated as reliable for transformation experiments (Yuen et al., 2008). Nevertheless, most successful insect transgenesis approaches have so far employed microinjection. For each species a specific microinjection protocol must be established that

allows the delivery of enough transgene DNA, but does not kill the embryos or sterilize them due to leakage of germ plasm (O'Brochta and Atkinson, 2004). It will very much depend on the sturdiness of the embryo whether such protocols include dechorionation, the use of oil or water immersion, fixation with glue etc. (Handler and James, 2000). Also the tip shape of the injection needle might have to be adjusted (Miller et al., 2002).

Choosing a genetic vector system. Pantropic retroviral vectors have been used to generate transgenic zebrafish (Lin et al., 1994), but in insects such vectors have so far not successfully been employed for germ-line transformation (Burns, 2000; Jordan et al., 1998). To date the most successful approaches have all used broad-range transposable elements. These have the advantage that DNA can be applied by itself and does not need to be packaged into potentially infectious viral particles. The most promising transposons currently available are the *hAT* family element *Hermes* (Subramanian et al., 2009; Warren et al., 1994), the *mariner* element *Mos1* (Medhora et al., 1988), the *Tc1/mariner* element *Minos* (Franz and Savakis, 1991), and the *TTAA*-specific element *piggyBac* (Cary et al., 1989), which have been used to successfully transform different insect orders (Atkinson et al., 2001; Handler, 2001; Handler and James, 2000; Horn et al., 2002; Pavlopoulos et al., 2007).

Since not every element will work in every species, it is recommended to try different transposable elements from various transposon families. The presence of a similar element might suppress transposition (Misra and Rio, 1990) or cause instability of a potential transgene insertion due to remobilization (Sundararajan et al., 1999). The presence of homologous transposable elements can be identified by low stringency southern hybridization or PCR (Bonizzoni et al., 2007; Handler et al., 2008; Lohe et al., 1995a, b; Zimowska and Handler, 2006). However, the presence of related transposable elements does not necessarily lead to repression of transposition or to the instability of transgenic lines (Handler and McCombs, 2000), since these elements might not be functional any more. To test for cross-mobilization activity simple plasmid-based excision assays should be performed (Atkinson et al., 1993). In the case of long generation times or limited amounts of embryos, the different transposons can be functionally evaluated in transposition assays before labor- and time-consuming germ-line transformation experiments are undertaken (Horn and Wimmer, 2000; Sarkar et al., 1997).

Choosing a transformation marker. The first efficient genetic transformations used marker genes that allowed transformants to be identified through rescue by the transgene of a mutant phenotype, such as eye color (Rubin and Spradling, 1982). Such "mutant-rescue" transformation markers can in principle be generated for any species. Although straightforward, this procedure is time consuming and risky. Moreover, mutant-rescue transformation systems are usually species-specific and the same considerable investment is required for every new species (Handler and James, 2000). Thus, to apply transgenic technologies to many insect species, the development of novel non-species-specific marker systems that allow easy and reliable identification of transgenic individuals was required.

Because of the random integration characteristics of transposon-based germ-line transformation, a marker should allow for reliable detection at greatly varying

expression levels. Moreover, the marker should be dominant and visible in wild-type backgrounds. To develop such markers, several research groups independently chose the gene encoding the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Prasher et al., 1992). In fact, the first fluorescent transformation markers were based on the more soluble, red shifted "enhanced GFP" (Catteruccia et al., 2000; Handler and Harrell, 1999; Horn et al., 2000; Pinkerton et al., 2000), which fluoresces 35-times more intensely than wild-type GFP (Cormack et al., 1996; Yang et al., 1996a, b).

Furthermore, because of the absence of marked "balancer" chromosomes (Lindsley and Zimm, 1992) in non-model insects, the development of genetic multi-component systems – such as binary expression or insertional mutagenesis systems – needs distinguishable transformation markers to identify separate transgene constructs independently. For this purpose distinguishable fluorescent transformation markers have been established, which are based on the yellow (EYFP) and cyan (ECFP) spectral variants of EGFP (Horn and Wimmer, 2000) and the red fluorescing protein DsRed (Handler and Harrell, 2001a; Horn et al., 2002; Nolan et al., 2002).

To reliably detect single copy insertions of transgenes, the detectable marker needs to be driven by either a strong constitutive or a tissue-specific promoter. In both situations, EGFP-based transformation markers have proven to be reliable and highly sensitive (Handler and Harrell, 1999; Horn et al., 2000). Constitutive promoters provide the advantage of allowing selection of transformants at all embryonic, larval, and adult stages. The constitutive *D. melanogaster* promoters of the genes *polyubiquitin* and *actin5C* were successfully used to drive EGFP expression and to identify transformants in several dipteran species (Fig. 9.1; Allen et al., 2001; Catteruccia et al., 2000; Handler and Harrell, 1999, 2001b; Heinrich et al., 2002;

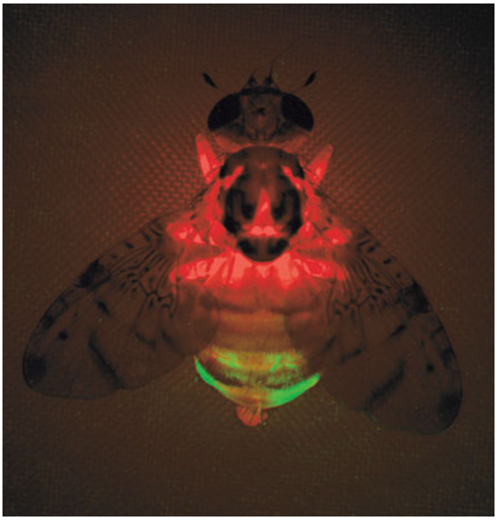


Fig. 9.1 Transformation markers. Transgenic medfly male carrying two fluorescent markers for visualizing transformation success and potentially enhanced marking during the monitoring process in a SIT program. A green fluorescent sperm marker and red fluorescent body marker are shown (Schetelig et al., 2009b; Scolari et al., 2008)

Heinrich and Scott, 2000; Horn et al., 2002; O'Brochta et al., 2000; Perera et al., 2002; Pinkerton et al., 2000; Schetelig et al., 2009a, b; Scolari et al., 2008). For the first stable germ-line transformation of lepidopteran species the *Bombyx mori actin A3* gene promoter was chosen (Peloquin et al., 2000; Tamura et al., 2000). However, transformation markers based on constitutive promoters have so far only been applied to closely related species, and it is questionable if such promoters can be functional across a wide range of insect orders. Natural promoters are usually complex; this means that, to be fully active in several divergent species, all trans-acting factors of such a natural promoter need to be evolutionarily conserved. This is less likely the more complex the promoter is structured.

To establish a widely applicable, potentially "universal" transformation marker, Berghammer et al. (1999) established a short and hyperactive promoter that responds to a single evolutionarily conserved transcriptional activator: an artificial DNA-sequence containing three binding sites for Pax-6 homodimers in front of a TATA box (3xP3; Sheng et al., 1997) drives strong expression of EGFP in the eyes of insects of three different orders (Berghammer et al., 1999; Hediger et al., 2001; Horn and Wimmer, 2000; Ito et al., 2002; Kokoza et al., 2001; Kuwayama et al., 2006; Marcus et al., 2004; Thomas et al., 2002). The "master control" function of the transcriptional activator Pax-6 in eye development (Halder et al., 1995) and its evolutionary conservation (Gehring, 2001) suggests that the 3xP3 promoter will be active in the eyes of many animals as has been demonstrated for the flatworm *Girardia tigrina* (Gonzalez-Estevez et al., 2003). In larval stages, 3xP3-EGFP mediates fluorescence in larval eyes and also in the central nervous system (Horn et al., 2000), which is also evolutionarily conserved between Diptera, Lepidoptera, and Coleoptera (Hediger et al., 2001; Ito et al., 2002; Kokoza et al., 2001; Kuwayama et al., 2006; Thomas et al., 2002). This might allow the application of 3xP3-EGFP also in insect species with eyeless larval stages.

Insect germ-line transformation. For the stable integration of a transgene construct, usually a donor and a helper plasmid are used, of which neither is functionally autonomous. The donor plasmid carries within functional terminal inverted repeats (TIR) the transgene of interest and a visibly detectable transformation marker. The helper plasmid encodes the transposase enzyme but cannot transpose itself, because the flanking TIRs are missing or defective. The co-injection of capped mRNA instead of the helper plasmid has been shown to improve the transformation efficiency in some cases (Pavlopoulos et al., 2004). In most successful insect transgenesis approaches microinjection of early embryos is employed by which the plasmids are deposited into the germ plasm of the syncytial blastoderm stage in the region where the primordial germ cells form and some of their nuclei will take up the plasmids. During development to an adult, the expressed transposase will recognize the respective TIRs and mediate transposition of the transgene construct from the donor plasmid onto a chromosome. Thus, some of the germ-line cells contain genomic transgene insertions. When such an insertion is transmitted to the offspring, the individual receiving the insertion is stably transformed. Since the efficiency of genomic transgene insertion is low, only a reliable and easily detectable transformation marker will make the efficient identification of the transgenic individuals possible.

The broad applicability of transformation systems is not restricted to established model systems and will therefore make functional genetic studies possible in a series of different insect species, which will facilitate comparative zoological and functional evolutionary studies (Wimmer, 2003). Moreover, the fact that fluorescent transformation markers can be applied to competitive wild-type strains rather than potentially labile mutant lines will be of particular interest for economically and medically relevant applications (Wimmer, 2003). In the remainder of this chapter, we will now address potential applications of insect transgenesis (1) in basic science to gain further knowledge of biological processes and (2) in insect pest management to potentially improve the SIT.

9.3 Basic Science: Tools for Functional Gene Identification and Characterization

Transgenesis is an important prerequisite, but with the exception of enhancer analyses, it does not provide many functional approaches by itself. To fully characterize gene functions, our aim must be to transfer as much transgenic technology to non-model insects as possible. The magnificent toolbox available to the *Drosophila* geneticist, with methods such as gene misexpression or insertional mutagenesis, will be an excellent source to draw from.

Systems for gene misexpression. The ectopic expression of a gene in cells, tissues or at developmental stages in or at which it is normally not active has proven to be an effective approach to identify its function by observing the phenotypic consequences. However, these consequences are often harmful and therefore necessitate that the misexpression is induced conditionally. One way to introduce conditionality is by using promoters derived from heat shock-inducible genes. Another way is to create binary expression systems, which allow ectopic expression only when two separate transgenes are present.

To cause heat shock-inducible gene expression, the most commonly used insect promoter is derived from the *D. melanogaster hsp70* gene (Lis et al., 1983). It has been employed for numerous ectopic expression experiments in *Drosophila* and its functionality has been demonstrated in transgenic strains of the silkmoth *B. mori* (Uhlirova et al., 2002) and the butterfly *Bicyclus anynana* (Ramos et al., 2006), which suggests that it might be also applicable to other insect species and orders. Nevertheless, specific heat shock protocols will probably have to be worked out for different species independently due to variable temperature requirements. Moreover, endogenous heat shock promoters might serve better than heterologous promoters from other species as has been demonstrated in the Mediterranean fruitfly *Ceratitis capitata* (Kalosaka et al., 2006, 2009). In the red flour beetle *Tribolium castaneum*, the *D. melanogaster hsp70* promoter works very poorly and in an insertion site- and partially stage-dependent manner, whereas an endogenous heat shock promoter shows high inducibility at all developmental stages (Schinko et al., 2010).

The heat shock can be used to induce high levels of gene expression at clearly defined time points. However, ectopic expression occurs in all cells and side effects

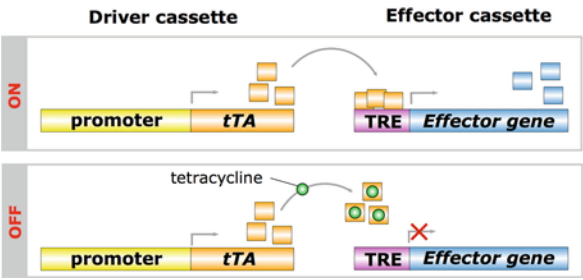


Fig. 9.2 Binary expression systems. All binary expression systems consist of two components: (i) a driver, which is able to activate an (ii) effector cassette to generate the intended reaction. For applied transgenic systems the most popular binary expression system is the Tet-Off system (Gossen and Bujard, 1992). The tetracycline-controlled trans-activator tTA mediates gene expression by binding to the tTA-response element (*TRE*). The main advantage of this system is that targeted gene expression can be switched off by food additives such as tetracycline or derivates thereof (Bello et al., 1998), since tetracycline and tTA form a complex that prevents tTA from binding to its response element

may hamper phenotypic analysis in the desired tissues. The non-targeted nature of heat shock-driven gene expression can be overcome by laser-induced heat shocks, which make it possible to target induced gene expression to single cells (Halfon et al., 1997) or to particular patterns (Ramos et al., 2006).

For targeted gene expression, binary expression systems enable the selective expression of any cloned gene in a variety of defined cell- and tissue-specific patterns. To avoid dominant lethality or sterility that can be caused by the ectopic expression of genes, these systems are made inducible by splitting driver and effector activities into two separate, benign constructs (Fig. 9.2). In the driver construct, a tissue-specific enhancer controls the expression of a heterologous transactivator, which is of yeast or bacterial-viral origin and nonharmful to the transgenic organism. In the effector construct, the gene of interest is regulated by a heterologous trans-activator-controlled promoter. The individual transgene constructs can be separately introduced into an organism's genome and only after crossing the respective strains, ectopic expression is induced in the progeny.

The most popular binary expression system in *D. melanogaster* is based on the yeast trans-activator GAL4 and the corresponding upstream activation sequence (UAS) (Brand and Perrimon, 1993). This system has been adapted to many uses and has made *D. melanogaster* one of the most genetically tractable multi-cellular organisms (Duffy, 2002). The GAL4/UAS system has so far been functionally transferred to the silk moth *B. mori* (Imamura et al., 2003) and *T. castaneum* (Schinko et al., 2010). Whereas the *Drosophila* system could be directly used in *B. mori*, for its use in *T. castaneum* all basal promoters needed to be exchanged with endogenous ones. In a variation of this system the activation domain of GAL4 has been fused to the DNA-binding domain of the bacterial LexA protein to create the trans-activator LexGAD. The corresponding activation sequence (LL) enables the construction of distinct effectors (Szuts and Bienz, 2000).

The tetracycline-controlled trans-activator tTA (Gossen and Bujard, 1992) is based on a bacterial-viral fusion protein and mediates gene expression by binding to the tTA-response element (*TRE*). The major advantage of this system is that targeted gene expression can be additionally controlled by a food supplement (Bello et al., 1998). Tetracycline and tTA form a complex that prevents tTA from binding to its response element, which therefore becomes inactive. Supplementing the diet with tetracycline makes it possible to switch off the system and thus allows a control beyond that imparted by the tissue-specific promoter. On the basis of the tTA/TRE, several transgenic systems have been created for *D. melanogaster* (Heinrich and Scott, 2000; Horn and Wimmer, 2003; Thomas et al., 2000), *C. capitata* (Fu et al., 2007; Gong et al., 2005; Schetelig et al., 2009a) as well as the malaria mosquito *Anopheles stephensi* (Lycett et al., 2004) and the yellow fever mosquito *Aedes aegypti* (Phuc et al., 2007). Since GAL4- and tTA-based expression systems are functional in mouse (Lewandoski, 2001), it is likely that these binary systems can be transferred further to other insects. However, the requirement of defined enhancers to drive the heterologous transactivators might actually pose a problem to employ or even to test these systems in non-model insects. For a first evaluation on how well the different systems work, it might be possible to use the artificial promoter 3xP3 to comparatively test such systems (Viktorinova and Wimmer, 2007).

Gene "knock-down" by RNAi. RNA-mediated interference (RNAi) causes post-transcriptional gene silencing and has revolutionized the analysis of gene function in non-model organisms, since it provides a powerful alternative to traditional genetics (Fire et al., 1998; Hannon, 2002). RNAi is based on sequence-specific mRNA degradation and translational repression, which is catalyzed by short fragments of double-stranded RNA (dsRNA; Hammond et al., 2001). In *D. melanogaster* and *T. castaneum*, RNAi is able to phenocopy lack-of-function mutants (Brown et al., 1999; Bucher et al., 2002; Kennerdell and Carthew, 1998; Tomoyasu et al., 2008). Besides *T. castaneum* and *D. melanogaster,* RNAi-induced phenotypes in insects have been reported for the Mediterranean fruit fly *C. capitata* (Pane et al., 2002; Salvemini et al., 2009), the tobacco cutworm *Spodoptera litura* (Rajagopal et al., 2002), the silkmoths *Hyalophora cecropia* (Bettencourt et al., 2002; Terenius et al., 2007), *B. mori* (Quan et al., 2002), the honey bee *Apis mellifera* (Beye et al., 2002; Gempe et al., 2009), and the milkweed bug *Oncopeltus fasciatus* (Angelini et al., 2005; Hughes and Kaufman, 2000b) and the method is used almost throughout the animal kingdom as a standard method for analyzing gene function (Matzke and Birchler, 2005).

The dsRNA is routinely applied by microinjection into embryos, larval instars, or pupae, depending on the stage in which the gene function was supposed to be analyzed. Since the introduced dsRNA only transiently interferes with gene expression, RNAi is not stably inherited. Thus, in order to obtain inheritable effects, transgenes with dyad symmetry have been constructed which produce hairpin-loop RNA when expressed (Dai et al., 2007; Fortier and Belote, 2000; Kennerdell and Carthew, 2000). The expression of these inheritable RNAi transgenes, however, needs to be conditional and therefore binary expression systems (Dietzl et al., 2007) or heat-inducible systems are used (Dai et al., 2007).

Targeted expression systems and RNAi gene knock-down will enable comparative phenotypic analyses of orthologous gene functions in diverse insect species. Therefore, we no longer need to rely on expression patterns alone for inferring gene functions, but can now functionally explore diversified biological processes as well as fascinating evolutionary events (Hughes and Kaufman, 2000a).

Site-specific recombination. As described above, insect transgenesis is mostly based on random integration of DNA fragments embedded into non-autonomous transposable elements. However, once a random insertion into a specific location of the genome has been identified as particularly useful in respect to transgene expression, ability to make the insertion homozygous, and lack of fitness costs, one would like to take advantage of that location for further modification and improvement. In this respect, site-specific recombination systems such as the bacteriophage P1-derived Cre ("Causes recombination"), the yeast-derived Flp ("flipase"), or the phage *phiC31*-derived Int ("integrase") have been used successfully to target transgenes at specific genomic sites (Wimmer, 2005b). The *phiC31* Int catalyzes the recombination between two different attachment sites, *attB* (present in the bacterial genome) and *attP* (present in the phage genome) (Sadowski, 1986). After recombination-mediated integration, two hybrid attachment sites *attR* and *attL* are generated, which are themselves no longer target sites for Int and therefore the integration is stable. This system has been used to generate transgenic *D. melanogaster*, *Ae. aegypti*, and *C. capitata* lines, after inserting the *attP* site in the fly genome by transposon-mediated transgenesis, and using a plasmid carrying the *attB* site (Bischof et al., 2007; Groth et al., 2004; Nimmo et al., 2006; Schetelig et al., 2009b). However, by this single site integration the complete plasmid including its antibiotic resistance is integrated. This is not ideal for release applications since the transgenic insects should only carry the minimum necessary transgene content and be free of any drug-resistance markers (NRC, 2002).

An effective alternative to the single site integration is provided by the recombinase-mediated cassette exchange (RMCE), which is based on the concomitant recombination at two target site variants that are introduced in the insect genome randomly by transposable elements. The variants must both be recognized by the same recombinase, but need to be ineffective in cross-recombination, which would otherwise cause excision of the cassette (Baer and Bode, 2001). By using the Cre recombinase in combination with an original *loxP* target site and a heterospecific variant thereof, RMCE has been demonstrated in *Drosophila* and shown that it can be used for comparative functional analysis of slight enhancer modifications (Oberstein et al., 2005). In a similar attempt, also FLP recombinase was shown to be effectively employed for RMCE in *Drosophila* when using the original target site FRT in combination with a heterospecific mutated variant (Horn and Handler, 2005). The availability of RMCE for *Drosophila* and, more generally, for insect transgenesis, is a major advance, as it will allow more detailed comparative analyses of transgenes. For future approaches intended to fight insect pests by the SIT, it will be of particular interest to identify genomic loci at which integration does not cause any fitness costs to the organism (Irvin et al., 2004). The ability to insert transgenes again and again at these particularly well-suited genomic target sites will

be a great aid to improving these strategies. Thus, targeted genomic insertion will advance the qualitative and quantitative functional comparison of similar transgenes and provide suitable integration points for transgenes of applied interest.

Isolation of novel gene functions. Gene functions that have been identified using chemical mutagenesis screens pose the basic difficulty of how to obtain molecular information on the gene that is affected. In contrast, insertional mutagenesis facilitates the cloning of a mutated gene through the recovery of genomic sequence surrounding the transposon tag (Huang et al., 2000). The efficient method of insertional mutagenesis relies on the development of two elements: one element, called "jumpstarter," encodes a transposase that mobilizes a second element, called "mutator," which integrates randomly into the genome and thereby causes mutations (Cooley et al., 1988). Fundamental to this approach is the use of two different types of transposons that cannot cross-mobilize each other (Horn et al., 2003).

Furthermore, transposon mobility also provides a way to sample the genome for enhancer activities (O'Kane and Gehring, 1987). Insertional mutagenesis and enhancer detection can actually be performed simultaneously in the same screen, as long as the mutator element contains a reporter gene driven by a basal promoter. When the mutator element integrates near a specific enhancer element, the basal promoter will respond and reporter gene expression will be driven in an enhancer-dependent pattern. In this way, genes can be identified that are specifically active in certain developmental processes or specific biological tissues. Moreover, by using a heterologous transactivator (Fig. 9.2) as a primary reporter, the insertion will become a tool for tissue-specific expression studies (Bello et al., 1998; Brand and Perrimon, 1993). Enhancer activities will then be identified by the use of a third element, called "reporter," in which a secondary reporter gene is regulated by the heterologous trans-activator-controlled promoter (Hacker et al., 2003; Horn et al., 2003). This elaborate insertional mutagenesis system provides the advantage that isolated enhancers can directly be used to express any cloned gene in the particular embryonic, larval, or adult tissues. Thus, with the help of a respective effector construct (Fig. 9.2), the effects of ectopic expression on the tissues can be examined. The isolation of *cis*-regulatory elements that direct stage-, tissue- or sex-specific gene expression will be of particular interest for applied transgenic approaches regarding insect pest management or the control of human disease transmission.

A non-species-specific insertional mutagenesis and enhancer detection system has recently been developed (Horn et al., 2003), which is based on the transposable element *piggyBac* (Cary et al., 1989). To independently identify the different type of elements, three distinguishable fluorescent markers (Horn et al., 2002; Horn and Wimmer, 2000) were chosen, that allow crosses of insect strains carrying the different elements without the need of balancer chromosomes. Moreover, the visible markers will facilitate stock keeping of mutant and enhancer detector lines. Since all components are derived from broad-range transposable elements and transformation markers, the insertional mutagenesis and enhancer detection system could be successfully transferred to *T. castaneum* (Trauner et al., 2009) and might be usable in a wide variety of non-model insects (Horn et al., 2003). Recently also for *B. mori*, an enhancer trap system has been established (Uchino et al., 2008).

Applying insertional mutagenesis to non-model insects will make possible the genetic analysis of insect-related biology that cannot be studied in *D. melanogaster*: This will include seasonal rhythms, diapause, defensive secretions, cast formation, altruistic behaviors, or mosquito-relevant issues like hematophagy and host-seeking. However, to be effective, this approach will still be restricted to organisms that are easily grown in the laboratory and have reasonably short generation times. Over time, it might actually be possible to establish a model organism for each major insect order. Then gene functions could be first identified in these species and more particular questions could be addressed by RNAi experiments after cloning the homologous genes from the specific species of interest.

Functional insect genomics. Insertional mutagenesis and enhancer detection enable direct correlation of sequence data with biological functions and will therefore be an integral tool for successful functional genomics in insects. For *D. melanogaster* and 11 other Drosophilids the genome sequence and its annotation are completed (Adams et al., 2000; Clark et al., 2007). Several other insect genomes have also been sequenced – including the malaria mosquito *Anopheles gambiae* (Holt et al., 2002), the yellow fever mosquito *Ae. aegypti* (Nene et al., 2007), the honeybee *A. mellifera* (Consortium, 2006), the red flour beetle *T. castaneum* (Richards et al., 2008), and forty genomes of domesticated and wild silk moths (Xia et al., 2009). The sequencing of several insect genomes like the jewel wasp *Nasonia vitripennis* or the pea aphid *Acyrthosiphon pisum* has been started (information can be found on the "*Baylor College of Medicine/ Human Genome Sequencing Center*" website) and many more will follow (Wu et al., 2009). Nonetheless, the biological functions corresponding to those sequences will have to be determined by transgenic approaches.

9.4 Insect Pest Management: Transgene-Improved Sterile Insect Technique

The availability of reliable and stable transformation systems will not only provide important tools for basic research but can also be applied for pest management; for example, to control insects that heavily damage agriculture and forestry or transmit deadly diseases to animals and humans. Genetic control based on the SIT (Dyck et al., 2005b) uses the release of mass-reared, sterile insects to cause infertile matings that reduce the pest population level (Fig. 9.3.; Klassen and Curtis, 2005; Knipling, 1955; Krafsur, 1998). The SIT is considered an environmentally friendly alternative to insecticides for insect species that can be mass reared in artificial settings. The SIT has been successfully employed in area-wide approaches to suppress or eradicate pest insects such as the pink bollworm *Pectinophora gossypiella* in California (Henneberry, 2007), the tsetse fly *Glossina austeni* in Zanzibar (Vreysen, 2001), the new world screwworm *Cochliomyia hominivorax* in North and Central America (Wyss, 2000), and various tephritid fruit fly species in various regions of the world (Klassen and Curtis, 2005). The sterilization process by ionizing radiation



Fig. 9.3 Principle of the sterile insect technique. When monitoring indicates that endemic insect pest levels start rising to become an economic problem or that foreign insect pests have been introduced (*left side*), sterilized male flies are mass released in an area-wide approach by airplanes or trucks (*right*). The more numerous sexually sterile males will mate with wild-type females resulting in infertile matings and subsequently lower crop damage. The pest level will decrease and when continued over several generations the pest can be eradicated from the area. Figure was generated by Programa Moscamed, Guatemala (Dyck et al., 2005a) (from Sterile Insect Technique, Principles and Practice in Area-Wide Integrated Pest Management, 2005, p 555)

decreases the competitiveness of sterilized males and thus large numbers of insects are required to inundate the pest population. SIT programs usually require male-only releases, but both sexes are needed for the rearing process. However, it is not just expensive to rear large numbers of "useless" females, but also detrimental to release large numbers of females into the environment, sterile or not, especially in the case of species that sting fruit with ovipositors, or transmit diseases by biting domestic animals and humans (Franz, 2005). Moreover, for the Mediterranean fruit fly (medfly) *C. capitata*, male-only releases have been shown to increase effectiveness of the SIT (Hendrichs et al., 1995).

At least three different types of traits can be biotechnologically introduced into insect strains to improve their use in the SIT (Robinson and Franz, 2000): first, a female specific lethality system could allow for efficient genetic sexing and the subsequent male-only release; second, a system that causes lethality after transmission to the progeny could replace the irradiation procedure; and third, marker systems could enable discrimination of released and naturally occurring insects.

Sexing strains. Separating males and females during the mass-rearing process of several insects for male-only releases has been shown to be very effective (McInnis et al., 1994; Rendon et al., 2004, 2000). This, however, can be labor-intensive if external morphology and hand sorting are used for sex-separation as is practiced for *G. austeni*, the tsetse fly. New knowledge about the variation in the developmental rate of tsetse sexes led to a sex separation system based on the timing of adult emergence, which eliminated the laborious hand-sorting (Opiyo et al., 1999, 2000). In the Oriental fruit fly *Bactrocera dorsalis* (McInnis et al., 2005) and the melon fly *Bactrocera cucurbitae* (McInnis et al., 2004) pupal color separation systems were developed and are used now to separate the sexes. In the Mediterranean fruit fly several different markers are available, but most of them are not ideal for large-scale SIT applications. Current medfly genetic sexing strains (GSSs) carry two mutations, the *white pupae* (*wp*; Rössler, 1979) and the *temperature-sensitive lethal* (*tsl*; Franz et al., 1994). The big advantage of these GSSs over marker-only sexing strains in medfly is the simple sexing by heat shock without the need for separation. Via heat shock all females of these GSSs are killed during early embryogenesis, but at the same time males are maintained. Thus, the mass rearing of medfly, with a possible weakly production of 4,000 million males, was tremendously improved by the introduction of GSSs (Franz, 2005). Nevertheless, only a maximum of 70% of total males survive during the sexing procedure in GSSs due to the mutations and chromosome segregation in the optimized GSS *Vienna 8* strain (Gerald Franz, Seibersdorf, Austria, personal communication).

Transgenic sexing systems that are based on the female-specific expression of a conditional lethal gene were first developed and tested in *D. melanogaster* (Heinrich and Scott, 2000; Thomas et al., 2000). In both studies, female lethality was made conditional by using the binary tTA-expression system, which can be suppressed by supplementing the food with tetracycline. Recently, a transgenic sexing system for medfly was generated using a similar tetracycline suppressible approach, but sex-specificity comes from a female-specifically spliced intron from the *transformer* gene which was used in an autocidal expression loop (Fu et al., 2007). With this system it is possible to kill 99.9% of the medfly females. On the one hand this system might be able to produce more viable males than existing GSSs, but on the other hand the majority of the female lethality occurs at late larval stages and the strains are not completely infertile in matings with wild females.

In a different approach, a male-specific promoter was used to drive expression of alcohol dehydrogenase to allow male medflies to survive alcohol treatment whereas females were supposed to die. However, the generated difference in alcohol tolerance was not sufficient to allow effective genetic sexing (Christophides et al., 2001).

Pane et al. (2002) have developed a clever approach that does not kill the females but actually turns them into males. RNAi-mediated knockout of the *transformer* gene in medfly hinders the establishment of an auto-regulatory mechanism necessary for female development and as a consequence the male pathway is taken. So far, this has been achieved by dsRNA injection. If the same can be effectively accomplished by transgenic RNAi, this would provide an elegant and efficient way of producing males only.

Transgenic sexing systems of any pest species should be completely female-lethal or use sex reversal to males, because the hatching of female larvae during mass rearing in facilities increases the costs. To ensure that no genetic load is transferred, a sexing system like in medfly should be combined with any kind of 100% sterilization. Such combined sexing and sterilization procedures can be tested in laboratories, in contained large environments, and finally also in open field tests with no genetic load transferred.

Replacement of irradiation. Once a species is sexed during the mass-rearing process, the remaining males have to be sterilized. A sterile insect in the sense of SIT is defined as "an insect that, as a result of an appropriate treatment, is unable to produce viable offspring" (FAO, 2005). In the beginning of SIT programs chemosterilants and irradiation were used to sterilize insects. The efficiencies of both were similar (Flint et al., 1975) but since most of the chemosterilants, which are partially introduced into the environment with the release of the insects are carcinogenic, teratogenic, and/or mutagenic, the exposure of insects to ionizing radiation has become the method of choice for most species. Irradiators using cobalt-60 or caesium-137 have been designed to keep the radiation exposure and doses to workers "as low as reasonably achievable" meaning that there will always be constant low exposure in the range of recommended dose limits and a residual risk (Bakri et al., 2005). In addition it has been shown that irradiation is indirectly related to the fitness of the insects (Parker and Mehta, 2007; Pedigo, 2002). Other difficulties are insect species like some Lepidoptera, which produce progeny even after irradiation doses of 500 Gy (in comparison 60 Gy are sufficient to guarantee 100% sterility for screwworm). The existence of such species led to the development of genetic methods to induce sterility.

A first approach to cause reproductive sterility by transgene-based embryonic lethality without the need of radiation was successfully shown in the non-pest insect *D. melanogaster* (Horn and Wimmer, 2003). The system is based on the transmission of a transgene combination that causes embryo-specific lethality in the progeny. However, prior to possible applications such a system had to be established or transferred to key pest insects. In the medfly, transgenic strains were produced using an autocidal overexpression loop of the protein tTA, which led to lethality when transgenic males were mated to wild-type females (Gong et al., 2005). But the majority of the lethality for these medfly strains occurs at late larval stages which is disadvantageous compared to the embryonic lethality described for the transgenic *D. melanogaster* strains from Horn and Wimmer (2003). As larvae are still produced, larval damage to targeted food would still occur and transgenes could be transferred into the wild population due to pupal survival (Gong et al., 2005). The first transgenic 100% embryonic lethality system for the Tephritid pest species *C. capitata* that causes complete reproductive sterility without the need of radiation is described in Schetelig et al. (2009a). The use of newly isolated early embryonic promoters of medfly led to the intended 100% conditional embryonic lethality without larval hatching. This prevents larval damage to fruits and the introgression of transgenes into wild-type medfly populations.

Such transgenic reproductive sterility systems have the advantage of eliminating the irradiation process, a possible release of insects at any life-cycle stage and an

expected fitness benefit of transgenic males over radiated males. In addition, this first 100% embryonic lethal system could reduce costs if combined with a sexing system and successfully evaluated in mass-rearing and large field tests. Since broad-range transposon vectors and widely applicable transformation markers were employed, the examination of this system should be straightforward in other pest species for which germ-line transformation protocols have been established.

Transgenic marking. Discrimination between released sterile and wild insects is critical for monitoring the effectiveness of an ongoing SIT program. For this, effective methods to attract and trap insects of both sexes are needed and various trapping methods have been developed. Once insects are trapped, these data are used to calculate the ratio of released to wild insects and indirectly the success of the control program (Vreysen, 2005). During this monitoring process, it is important to easily recognize and differentiate released insects from wild ones. For this reason, released medfly males are dusted with fluorescent dye powders before release (Parker, 2005), but this method is expensive, labor intensive and error-prone (Hagler and Jackson, 2001; Robinson and Hendrichs, 2005). The transgenic introduction of a fluorescent transformation marker, which does not compromise survival or fitness, would enable the identification of released insects in a simple way (Peloquin et al., 2000). Fluorescent protein-based markers can be applied and detected in wild-type organisms. Moreover, spectrofluorometric assays have been established that provide sensitive and fast analyses for field detection after release (Handler, 2001, 2004; Handler and Harrell, 2001a, b).

Furthermore, in two mosquito species, *An. gambiae* and *Ae. aegypti*, genetically engineered fluorescent sperm-marking systems were established (Catteruccia et al., 2005; Smith et al., 2007). The fluorescence was limited to the testes of males and could therefore be used to differentiate between released and wild-type males and to identify the mating status of the wild-type females (Smith et al., 2007). A similar sperm-marking system with green or red fluorescent testes has recently also been developed in the medfly (Fig. 9.1; Scolari et al., 2008) as well as the caribfly *Anastrepha suspensa* (Zimowska et al., 2009), and the generated medfly strains showed their competitiveness to wild-type flies in first laboratory tests. The described marking systems can be used for direct monitoring in the field or in case of sperm-marking systems even for sexing males and females by automated cell sorters (Catteruccia et al., 2005).

The effective implementation of SIT against agricultural pest species and potential biotechnological improvements by insect transgenesis has also raised interest in application of SIT to control human disease vectors including several mosquito species (Alphey et al., 2010; Benedict and Robinson, 2003, 2008).

Species transfer of transgenic systems. Besides the simple transgenic marking, most of the mentioned approaches were originally tested in *D. melanogaster*. The first direct transfer to pest species demonstrated that *cis*-regulatory elements might not even work in closely related species (Schetelig et al., 2008). Studies on the evolutionary conservation of enhancers and promoters (e.g., from cellularization genes) will determine whether the transgene construct can directly be used in diverse species, or whether endogenous corresponding genes have to be isolated as has been successfully done for medfly (Schetelig et al., 2009a). Sex-specific

regulatory elements might not even function in closely related species (Hediger et al., 2004), since the sex-determining cascades evolve much more rapidly than other regulatory pathways (Schütt and Nöthiger, 2000), even though, sex-specific splicing introns seem to function across higher fly species (Fu et al., 2007). If no appropriate genes can be found based on homology or with tissue-specific differential display approaches (Schetelig et al., 2009a, 2007), enhancer detection screens should allow the identification of suitable *cis*-regulatory elements. But difficulties in engineering and transferring a transgenic system directly from one species to another due to low conservation of genes, also bear an advantage for the safety of using transgenes in field releases, because species-specific transgenic systems are of no risk to other insects even though a rare horizontal transfer might occur.

Transgene stability. A major concern for SIT programs using transgenic insects is the stability of the transgene in the field and during mass-rearing maintenance (Handler, 2004). Transgene instability can cause in different ways the loss of strain attributes important to the program and result in reduced effectiveness of the biocontrol program (Handler, 2004). In *D. melanogaster*, systems have been successfully tested, which stabilize a transposon vector by terminal sequence deletion (Handler et al., 2004; Horn and Handler, 2005). In *C. capitata*, a system creating *piggyBac*-mediated but transposon end-free insertions was established (Dafa'alla et al., 2006). Recently, a size-independent site-specific recombination system (Venken et al., 2009) was tested and used in *C. capitata* to modify *piggyBac*-mediated transgene insertions (Schetelig et al., 2009b). The modified strains have been tested for transgene stability in large populations and have been shown to be inert to a provided active transposase source (Schetelig et al., 2009b). The development of non-autonomous as well as remobilization-defective transposon insertions will increase the safety of SIT programs, when taking transgenic improvements of pest-management programs from the lab to the field (Wimmer, 2005a).

9.5 Ecological and Ethical Considerations

Before any release, great care must be taken to employ as many safety features as possible to prevent undesired spread of the transgenes (Handler, 2001). Laboratory studies will first have to assess transgene stability and fitness constraints in large populations (Ashburner et al., 1998; Atkinson, 2002). To achieve transgene stability, transposons should be nonautonomous and chosen so that no endogenous or related transposon activities are present in the species of choice (Schetelig et al., 2009b; Sundararajan et al., 1999). To further avoid rare cross-mobilization of the transgenes, vectors that enable effective immobilization by deletion or rearrangement of transposon ends should be developed (Handler, 2004). Moreover, the introduced transgenes must not contain positively selectable drug-resistance markers.

For first evaluations of the environmental impact of transgenic insects, SIT programs with their high quality control during the production of sterile insects will minimize the potential ecological concern the release of transgenic organisms might

bring about (Robinson and Franz, 2000; Robinson and Hendrichs, 2005). The sterility of the released insects will serve as a biological safety mechanism that impedes vertical transmission of the transgenes, which will be removed from the ecosystems with the cessation of the SIT program. Transgene constructs containing fluorescent transformation markers only will be suitable for the first field trials, since they will improve SIT applications by simplifying the monitoring (Peloquin et al., 2000), but do not provide advantages to the carrier organism and actually allow the identification of carriers at later stages. This will minimize the risk of an extremely rare but potential horizontal gene transfer. For the pink bollworm *P. gossypiella*, an important agricultural insect pest, the U.S. Department of Agriculture (USDA) and Animal and Plant Health Inspection Service (APHIS) permitted a confined field study of a transgenic EGFP-marked strain in 2006. The strain was released in Yuma County, Arizona, within a contained SIT program. The release was permitted, because an environmental assessment (EA) reached a finding of no significant impact (FONSI) to the environment. Besides this first attempt to test transgenic insects in a field study, regulations including the implementation of transgenic pink bollworm and fruit fly into agency pest control programs were filed with the Environmental Protection Agency of the United States and approved in 2009 (APHIS, 2009). These regulations will help to evaluate transgenic systems in the future.

Given the understandably intense public scrutiny and the general lack of knowledge on potential risks, all projects that require the release of transgenic insects into the environment need to be planned with utmost care. The initial position of using transgenic technology for SIT is rather different to the use of biotechnology in other areas. The developed systems for marking (Catteruccia et al., 2005; Scolari et al., 2008; Smith et al., 2007) and embryonic lethality (Schetelig et al., 2009a) do not carry positively selectable elements such as resistances and thus differ fundamentally for example from plant biotechnology, where transgenically produced insecticide (Tabashnik et al., 2006, 2008) or herbicide resistances are used (Gasser and Fraley, 1989). Another fundamental difference to transgenic plants is, that released sterile insects of SIT programs are reproductively sterile and therefore do not transmit their genetic background into wild populations. In this respect it has to be ensured that the released insects are completely sterile no matter whether sterilized by radiation or using autocidal control with transgenic technology. In addition, already at the initial stages of this methodology, molecular and population geneticists, entomologists, ecologists as well as pest-management specialists need to coordinate their efforts along with regulatory agencies and the public to establish a safe use of the great potential transgenic insects have to offer.

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Part III
Industrial Applications of Insect Biotechnology

Chapter 10
Insect Cells for Heterologous Production of Recombinant Proteins

Christoph Becker-Pauly and Walter Stöcker

Abstract Heterologous gene expression has become an indispensable and powerful tool for the production and subsequent functional analysis of proteins that are difficult to purify from their natural sources. Furthermore, it is the method of choice for the production of variants by introducing site-specific mutations into the DNA encoding the protein of interest. However, many systems are biased by disadvantages. The inability of bacteria to confer important post-translational modifications often results in functional failure of the recombinant protein. In addition, disulfide bonds are not formed properly in bacterial systems. Mammalian cells on the other hand modify properly, but they generally provide low product yields. Insect cells have become an extremely valuable alternative to these established systems. Several cell lines are in use mostly from butterflies and flies. These cells can be infected with insect-specific viruses encoding the desired protein or they are stably transfected with corresponding plasmids for continuous protein production. Besides comparably high product yields, insect cells do glycosylate secreted proteins at appropriate sites, with somewhat altered usage of sugar residues. This often helps to overcome solubility problems and may render the product more homogeneous.

Keywords Baculovirus · SF9 · SF21 · Schneider cells · Trichoplusia

10.1 Heterologous Protein Expression in Insect Cells – History

The use of insect cells for heterologous protein expression has become feasible after the successful synthesis of recombinant baculoviruses (*Autographa californica* nuclear polyhedrosis virus; AcNPV). The first report on the production of human interferon in butterfly cells was published in 1983 by Max Summers and

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colleagues from Texas A&M University (Smith et al., 1983). They presented a system, which allowed the expression of a gene of interest with the help of insect pathogenic viruses. The clue in their strategy was the exploitation of the fact that these baculoviruses physiologically produce polyhedrin, a protein nonessential for virus amplification, in amounts up to 70% of total cell protein (Hu et al., 1999; Jarvis et al., 1992).

Taking advantage of the strong polyhedrin promoter, the polyhedrin encoding viral DNA stretch was replaced by a cDNA for human interferon (Smith et al., 1983). This led to a yield of recombinant protein of about 5 mg/l of cell culture supernatant. Hence, the baculovirus expression system was successfully established.

In the meantime, several companies provide systems for protein expression via recombinant baculoviruses, which allows easy application of this system not only for virologists but also for a broad scientific community.

10.2 Insect Cells – Introduction

10.2.1 Types and Sources

Most of the over 500 established insect cell lines are derived from Lepidoptera and Diptera, whereas about 100 are from other insects and a fewer number from other invertebrates (Grace, 1962; Hink, 1972; Lynn, 2001). The most conventional insect cell lines are derived from the fall armyworm *Spodoptera frugiperda*, the cabbage looper *Trichoplusia ni*, and the fruit fly *Drosophila melanogaster*.

Different tissues can be used to establish stable cell lines, namely the endocrine system, the mid gut epithelium, the fat body, the muscle, the ovaries/testis, the cuticular epithelium, the nervous system, the imaginal discs and the hemocytes, as well as the whole embryo (Lynn, 1996). For example, the most common cell lines are derived from pupal ovaries of *S. frugiperda* (*Sf*9, *Sf*21) (Vaughn et al., 1977) and the embryos of *T. ni* (*Tn*5 or HighFive^{TM}) (Granados et al., 1986; Wang et al., 1994) and *D. melanogaster* [Schneider 2;S2 (Schneider, 1972) or Kc (Simcox et al., 1985)]. These cells can either be used for virus production or, after infection with baculoviruses or stable transfection with suitable plasmids, for recombinant protein production.

10.2.2 Post-Translational Modifications

Insect cells are eukaryotic and therefore capable of post-translational protein modification. Hence, even larger proteins composed of several individual polypeptide chains are commonly correctly assembled from their subunits, and in most cases proteins of mammalian origin are properly modified with respect to disulfide linkage, phosphorylation, fatty acid acylation and glycosylation. Interestingly, the glycosylation reactions occurring within the endoplasmic reticulum are highly

conserved among eukaryotes. In contrast, the reactions that take place in the Golgi complex are species specific and also cell type specific (Jacobs and Callewaert, 2009). It is still not completely understood whether failures in correct protein processing in certain cell lines are simply due to an overload of the protein synthesis machinery or to the lack of suitable enzymes (Laprise et al., 1998; Murphy et al., 1990). However, at least the numbers of glycosylating enzymes differ between species. For comparison, there are more than 300 different glycosyl enzymes in humans, but only about 100 in *Drosophila* (Yano et al., 2005). This would be consistent with the observation that *N*- and *O*-glycosylation in insect cells is less complex than in mammalian cells, resulting in shorter sugar trees. In some instances, the less complex *N*-glycosylation can be advantageous for example for protein crystallization to solve the three-dimensional structure. On the other hand, protein glycosylation might be important for physiological processes, for example the binding of a ligand to a receptor. To cope with this problem, the *Sf* SWT-1 insect cell line (derived from *Sf* 9) was developed that attaches terminally sialylated *N*-glycans to proteins of mammalian origin (Hollister and Jarvis, 2001; Hollister et al., 1998; Jarvis et al., 1996). "Glycoengineering" has become a prosperous research field for the production of tailor-made glycoproteins (Jacobs and Callewaert, 2009).

An example for successful production of a multi-domain protein with complex modifications in *T. ni* cells is the human metalloprotease meprin α. This enzyme builds huge oligomers with sizes in the mega Dalton range, thus being the largest secreted protease known (Fig. 10.1) (Becker et al., 2003; Becker-Pauly et al., 2007; Köhler et al., 2000). The oligomerization requires several post-translational

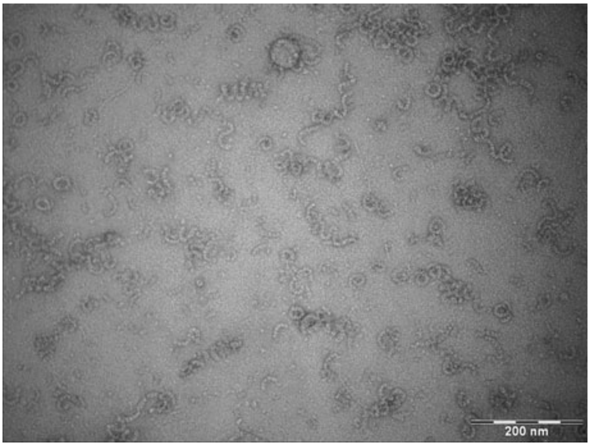


Fig. 10.1 The recombinant human metalloprotease meprin α, expressed in *Trichoplusia ni* cells (High5). The enzyme builds huge oligomeric complexes that are arranged in curved chains, circles and spirals, which may be wound up in spindle-like stacks. These meprin α oligomers reach sizes in the 6 mega Dalton range and, hence, are considered as the largest secreted proteinases known. The picture was kindly provided by Philipp Arnold (University of Mainz, Institute of Zoology, Molecular Animal Physiology)

modifications. The enzyme contains a C-terminal trans-membrane anchor, which is cleaved off during biosynthesis in the endoplasmic reticulum by a furin-like protease (Marchand et al., 1995). The oligomerization is a two-step process. First, disulfide linked dimers are formed, which then further associate by non-covalent interactions. The production of human meprin α demonstrates the potential of insect cells for the assembly of complex proteins, which is neither possible in bacteria nor comparable to mammalian cells regarding the yield of protein. Meprin α can be purified from cell culture suspension of High5 cells in a range of up to 5 mg/l.

10.3 Baculoviruses

10.3.1 Classification

The family of Baculoviridae is divided into the genera granuloviruses (GVs) and the nucleopolyhedroviruses (NPVs), with the latter being commonly used for heterologous production of recombinant proteins (Van Regenmortel, 2000; Zanotto et al., 1993).

All Baculoviridae contain a circular double stranded and supercoiled DNA molecule, the so-called bacmid, condensed within a nucleocapsid (Thiem and Miller, 1989; Wilson et al., 1987). These viruses build characteristic virions in a crystalline protein matrix called occlusion bodies (OBs), predominantly consisting of polyhedrin (NPVs) or granulin (GVs)(Van Regenmortel, 2000).

The most obvious difference between both genera is indeed the size of these occlusion bodies (OBs) and the number of virions. NPVs, like the *A. californica*, build a polyhedron with a diameter of about 1.0 μm that contains several virions, whereas the granules of GVs, are much smaller (about 0.3 μm in diameter) containing just one or two virions (Murhammer, 2007).

10.3.2 Structure and Replication

Polyhedrin, the major component of NPV occlusion bodies (OBs), is a 29-kDa protein that exhibits specific sequence motifs responsible for particle formation (Jarvis et al., 1992). The polyhedrin core of the OBs is surrounded by the polyhedrin envelope (PE) mainly consisting of the phosphorylated protein PP34 (Whitt and Manning, 1988).

This complex protects the virions (i.e. virus particles outside of cells) against physical and biochemical stress outside the host and shields them from proteolytic activity within the cells during infection (Whitt and Manning, 1988; Williams et al., 1989). Once the OBs are taken up by the host insect larvae (e.g. Lepidoptera) the polyhedron becomes solubilized in the alkaline milieu of the mid gut, thereby releasing occlusion-derived viruses (ODVs) (Fig. 10.2) (Federici and Hice, 1997; Flipsen et al., 1995; Horton and Burand, 1993). Thereafter, intestinal epithelial cells are

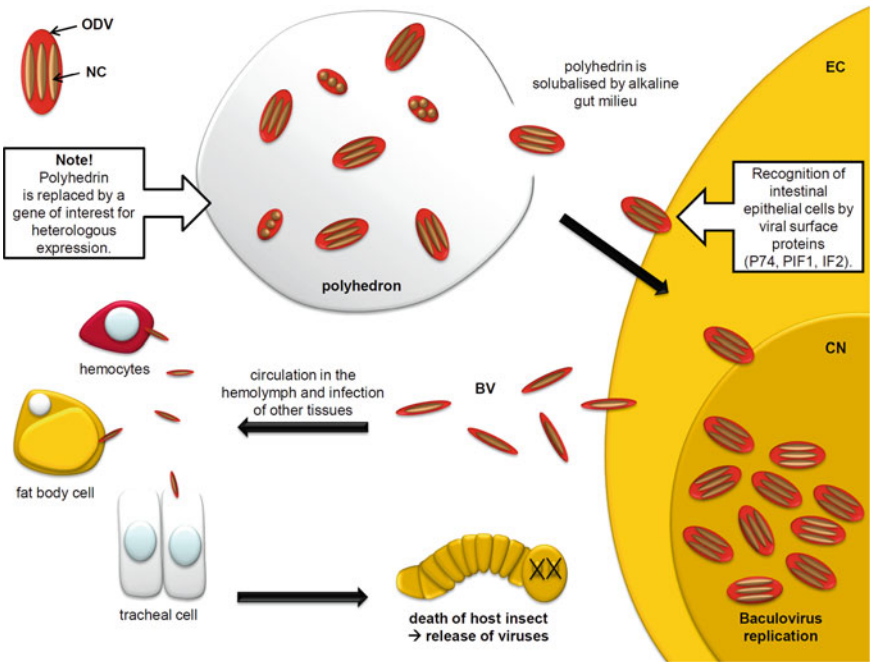


Fig. 10.2 Replication of baculoviruses in lepidopteran larvae. The occlusion-derived viruses (ODV) are embedded within the polyhedron. After solubilization in the midgut ODVs infect epithelial cells (EC) and replicate in the cell nucleus (CN). Budded viruses (BV) infect further tissues leading to the death of the larvae, subsequently releasing viruses into the environment. NC, nucleocapsid

infected, mediated by molecular interactions between virions and cell surface proteins (Faulkner et al., 1997; Kikhno et al., 2002; Pijlman et al., 2003). Replication of viruses occurs within the cell nucleus, resulting in budding viruses (BVs) in the initial phase of infection (Washburn et al., 2003). This viral phenotype circulates in the hemolymph and promotes further infection, predominantly in fat body cells, tracheal matrix, hemocytes, and the midgut (Federici and Hice, 1997). At advanced stages of larval infection, after 1–3 weeks, the production of BVs is changed to that of ODVs and polyhedra. The fragile cuticle of dead larvae allows for almost unhindered release of baculoviruses embedded in the polyhedra (approximately 10^{10} per larvae), into the environment (Entwistle and Evans, 1985).

10.4 Commercially Available Expression Systems

In the beginning of baculovirus protein expression, it was essential to separate recombinant from original parental viruses, since the frequency of recombination was quite low (<1%). With the help of new systems it became feasible to produce recombinant baculoviruses essentially free of parental stages, reducing the time it

previously took to identify and purify a recombinant virus from 4 to 6 weeks to within 7 to 10 days.

10.4.1 Bac-to-Bac® System (Invitrogen)

In order to use baculoviruses as expression vectors for recombinant protein production the gene of interest has to be inserted in close proximity to a potent promoter (mostly the polyhedrin promoter) (Smith et al., 1983), without influencing virus replication. Because of the large size of the baculovirus genome (about 134 kbp) (Ayres et al., 1994), containing a multitude of nuclease cleavage sites, it is not a practical method to directly integrate the cDNA of interest. Therefore, an enormous number of different transfer vectors has been developed, often commercially available through companies, but also from research institutes (Murhammer, 2007).

Such a transfer vector should comprise some general features: a promoter, a multiple cloning site for insertion of the coding gene, a transcription termination signal and a flanking region homologous to the viral DNA. The latter could be a bacterial transposon attachment site, which allows for DNA recombination via transposase activity (Invitrogen). Other beneficial features are sequences coding for a signal peptide, which allows for harvesting the secreted protein directly from the medium, and a suitable affinity tag for purification and detection. As an example, the metalloprotease meprin α expressed in High5 suspension cultures (Fig. 10.2) contains a signal peptide directing the protein to the extra-cellular space and a Strep-tag for affinity chromatography purification (Becker-Pauly et al., 2007).

A variety of baculovirus expression systems together with the appropriate transfer vectors are commercially available (Murhammer, 2007). For those starting in this field, it is recommendable to choose a complete package from a company including all essentials perfectly matched with each other.

The Bac-to-Bac® system from Invitrogen using a technology basically developed by Luckow and coworkers in 1993 (Luckow et al., 1993) was the first insect cell expression system on the market. This system provides a good example for the principles of heterologous gene expression using baculoviruses and will be explained here in some detail. But potential users might want to choose alternatives from other companies offering systems with interesting and innovative features (e.g. the *flash*BAC™ from Oxford Expression Technologies).

The principle of the Bac-to-Bac® system is based on the recombination of the *A. californica* nuclear polyhedrosis virus (AcNPV) with the help of a specifically designed transfer vector (Fig. 10.2). The expression cassette contained within the transfer vector can be inserted by site-specific transposition into a baculovirus shuttle vector (bacmid). The bacmid as well as the corresponding transfer vector (pFastBac), both propagated in *E. coli* cells (DH10Bac™), contain the attachment site for the bacterial transposon Tn7 (mini-attTn7). This, in combination with the transposase encoded on a helper plasmid, allows for DNA-recombination resulting in the transfer of the expression cassette into the bacmid.

Successful transposition interferes with the expression of the lacZ-gene present on the bacmid, leading to white colonies on a chromogenic substrate such as

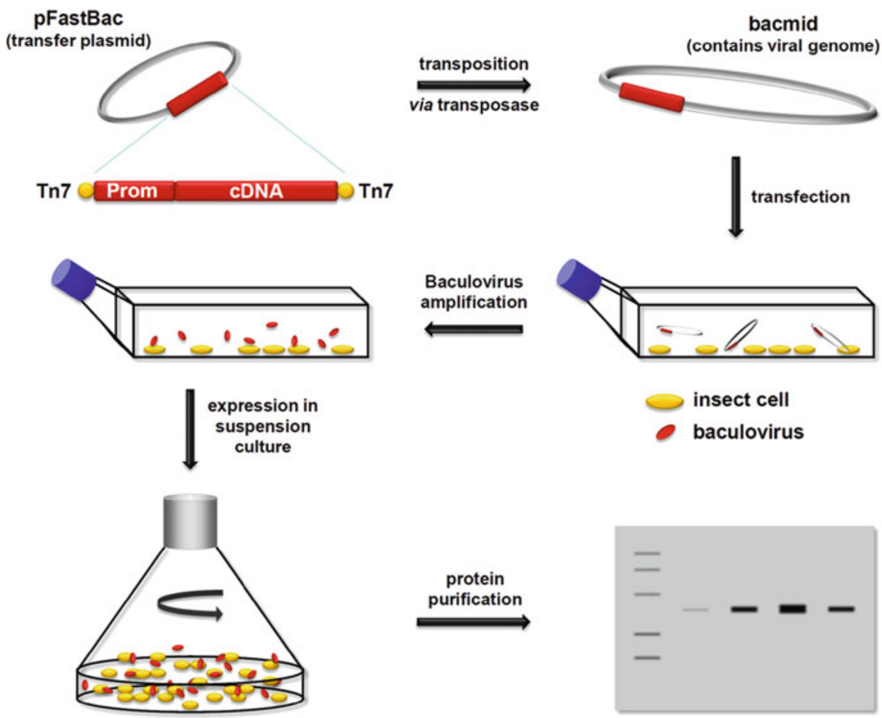


Fig. 10.3 The baculovirus expression system based on the Bac-to-Bac® system (Invitrogen). Prom, polyhedrin promotor; cDNA, heterologous DNA encoding the protein of interest; Tn7, transposase recognition site

Blue-gal or X-gal, while unaltered bacmids result in blue colonies. Thus, *E. coli* cells bearing recombinant bacmids can be selected for DNA preparation prior to transfection of insect cells (e.g. *Sf*9). After a recommended incubation time of 4–5 days to allow the virus to replicate, the baculovirus particles are harvested from the cell culture supernatant (Murhammer, 2007). The actual virus titer can be determined by the plaque-assay (Bachrach et al., 1957; Cooper, 1961; Murhammer, 2007). In order to achieve an appropriate titer (>10^7 pfu/ml; pfu = plaque forming units), several rounds of amplification may be required. Subsequently, infection of fresh cells (e.g. High5) enables protein production, followed by purification and analysis (Fig. 10.3).

10.5 Lab Facilities

In general, two routes are commonly used for heterologous expression. Adherent cells in plastic culture flasks are used on the one hand to amplify viruses on a larger scale and on the other hand to obtain protein from relatively low volumes of cell culture supernatant. To achieve higher yields, suspension cultures are used with a volume of up to hundreds of liters.

All necessary materials, like serological pipettes, plastic-, spinner-, Fernbach- and Erlenmeyer-flasks, laminar flow clean benches, media and others are provided by a broad range of companies and are not listed in detail here. Thermostatted incubators must keep stable temperatures of 27°C. With respect to the choice of orbital shakers it is very important that the throw turn is wide enough (50 mm) to ensure safe and gentle shaking of eukaryotic cells. For suspension culture, insect cells should be grown in Spinner-, Erlenmeyer- or Fernbach flasks (~0.1 l to 2.0 l) with gas permeable stoppers. To avoid cell damage flasks must not be equipped with mixing baffles. The use of additives, like Pluronic® F-68 (BASF), is recommended to prevent disruption of cells by shearing.

10.5.1 Cell Growth

The cell lines recommended to propagate baculoviruses in adherent cultures are *Sf*9 and *Sf*21, derived from the fall armyworm *S. frugiperda* (Vaughn et al., 1977). Although both cell lines are feasible for protein production, cells from the cabbage looper *T. ni* (*Tn*5 or HighFive™) are suggested to gain higher yields of recombinant protein (Wang et al., 1994).

Compared to most mammalian cells, the growth of insect cells in adherent cultures is simple and appropriate in any kind of cell culture flask, dish, or plate. To propagate the cells, they can easily be released mechanically from the surface with a pipette. Several companies offer a variety of cell culture media. For protein production, serum-free media are recommended, alleviating the purification.

Before starting with larger scale expression in suspension culture, it proved to be useful to initially optimize the multiplicity of infection (MOI) in adherently grown cells. This is simply achieved in 24-well plates, infecting the cells with various amounts of viruses and harvesting at different time points. Afterwards, the optimal conditions for the production of recombinant protein can be revealed by Western Blot analysis, if an appropriate antibody is available.

The large-scale expression of recombinant proteins in insect cells has been scaled up to industrial scale in bioreactors with volumes in the hectoliter range and strict control and constancy of physical conditions like temperature and oxygen supply.

10.6 Insect Cells for Continuous Protein Expression

Protein yields from baculovirus transfected cells are often up in the range of hundreds of milligrams per liter cell culture suspension (Caron et al., 1990; Neutra et al., 1992). Unfortunately, the expression of secreted or membrane-bound proteins is most often less efficient, probably due to adverse effects during secretion by the host cell (Jarvis and Summers, 1989). Therefore, a stably transfected *Sf*9 cell culture system has been developed (Jarvis et al., 1990). Here, the gene of interest is under control of a constitutively active promoter, for example derived from

the baculovirus immediate early gene *ie-1* (Guarino and Summers, 1986, 1987). For selection of positively transfected cells, an appropriate antibiotic resistance marker, like neomycin or hygromycin, has to be introduced additionally. A comparison between the expression of the human tissue plasminogen activator (tPA) with the baculovirus and the stably transformed system, revealed a better secretion of the protein for the latter, albeit the overall concentration was higher using the virus-infected cells (Jarvis et al., 1990). The constitutively secreted protein can be collected and purified from the supernatant of adherently grown cells, which usually yields lower amounts of recombinant protein compared to suspension cultures.

Cells from *D. melanogaster* are frequently used for constitutive secretion of recombinant proteins. The most common ones are *Drosophila* Schneider 2 (S2) (Schneider, 1972) and Kc (Simcox et al., 1985), both derived from embryonic tissues. Different promoters can be used in these cells, like the strong constitutive actin 5C promoter (Krasnow et al., 1989; Winslow et al., 1989) or the inducible (e.g. by addition of $CuSO_4$) metallothionein promoter (MT) (Bunch and Goldstein, 1989; Bunch et al., 1988). The choice of the insect expression system most suitable for the protein of interest might also be governed by the quality of post-translational modifications, in particular if proteolytic processing is desired. It has been observed that cleavage sites, which in mammalian cells are typically hydrolyzed by furin-like prohormone convertases, are insufficiently recognized in baculovirus-infected lepidopteran cells. Examples are the incorrect processing of the human metalloproteinase ADAM33 and of the human astacin metalloproteinase BMP1 (bone morphogenetic protein 1, i.e. the procollagen C proteinase). In both cases cleavage of furin sites was less efficient in a baculovirus lepidopteran system than in constitutively transfected *Drosophila* cells (Prosise et al., 2004; Wermter et al., 2007).

10.7 Protein Production in Larvae

Although large-scale protein production in insect cells is generally much cheaper than in mammalian systems, it is still expensive regarding all necessary cell culture materials and consumables. In this regard insect larvae, infected with baculoviruses, can serve as natural bioreactors, synthesizing the protein of interest in vivo. Meanwhile, this service is provided by several companies (e.g. Entopath or Chesapeake PEARL), promising 1–3 mg protein per larvae, harvested 2 days postinfection (Mathavan et al., 1995; Miyajima et al., 1987) at very low production costs. Moreover, in some cases a more efficient post-translational modification was observed compared to insect cell culture (Andersons et al., 1991; Yamada et al., 1990).

The first proteins were produced in larvae of the silkworm *Bombyx mori* [e.g. human α-interferon (Maeda et al., 1985) or mouse interleukin-3 (Miyajima et al., 1987)], followed by several other hosts (Ahmad et al., 1993; Gretch et al., 1991; Kuroda et al., 1989; Richardson et al., 1992). Also, *T. ni* larvae are commonly used commercially and for research purposes as shown for human interleukin-2 (Pham et al., 1999) and human adenosine deaminase (Medin et al., 1990).

10.8 Conclusions

Insect-cell-based expression systems are generally very attractive alternatives to heterologous expression in bacteria, yeast, or mammalian systems. They provide high protein yields at moderate costs. There are slight variations in complex glycosylation, which can be overcome by choosing appropriately complemented cell lines if necessary. Generally, shorter sugar trees provide the advantage of more homogeneous glycoprotein batches in highly expressing cell cultures.

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Chapter 11
Biotechnologies Based on Silk

František Sehnal

Abstract Natural silks are fibrous polymers built of several protein components. Commercial silk fibers are obtained from the cocoons of a handful of moth species by an ancient technology. The core proteins, which are responsible for filament formation in these silks, are called fibroins and the surface sticky proteins are sericins. Silk is still primarily used for textiles and related products but applications in cosmetics and medicine are of growing importance. Sericins have been commercialized as additives to tissue culture media and are being tested as scaffolds for tissue reconstruction. Modern transgenic technologies are used to obtain silk threads with new properties or to manufacture minor silk components. Controlled conversion of soluble proteins into filaments is the major problem in the development of recombinant silk fibers. Sericin-like recombinant proteins are promising for use in cosmetics, medicine, and industry.

Keywords Fibroins · Recombinant silk · Sericins · Sericulture · Transgenesis

11.1 Silk Use in Textiles and Related Products

11.1.1 Silk as a Natural Fiber

Man has learned how to use a variety of natural fibers. Fibers of plant origin are in principle polymers of sugars, while the wool and silk derived from mammals and arthropods, respectively, are protein polymers. Silks are produced by numerous arthropods and perform diverse functions. Spidermites and spiders are the best known silk producers of the arthropod subphylum Chelicerata. Spidermites stretch fine silk fibers close to their eggs as a form of protection against potential predators.

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The spiders have up to seven types of silk glands, each producing material of different properties and for different purposes. Some spiders use certain types of silk early after hatching for air-sailing, while later in life they employ other silks to spin webs to catch prey and to construct cocoons for egg protection. Harvesting natural spider silks is difficult but the method of transgenesis coupled with the synthesis of artificial genes has opened a door to the production of appreciable amounts of spider-like silks in diverse organisms (Vendrely and Scheibel, 2007).

Silk production is widespread in the subphylum Tracheata, above all in the class of Insecta (Sehnal and Craig, 2009). Males of some apterygote hexapodes stretch silk fibers as a clothes line onto which they hang their spermatophores to be picked up by females. Silk spinning from special glands in the tarsi of the front pair of legs is a typical feature of the webspinners (Embioptera). These pterygote hemimetabolic insects spend most of their life in silky tubes and use silk also for the protection of their eggs. Silk spinning occurs in the larvae of several orders of holometabolic insects: Hymenoptera (sawflies, ants, honeybees), Anoplura (fleas), some Diptera (water midges), Trichoptera (caddisflies), and Lepidoptera (caterpillars). All these larvae produce silk from a pair of large, tubular labial glands whose original function was the secretion of saliva. Gland outlets join into a single spinneret on the labium on the ventral side of the head. Silk is typically secreted at the end of larval development and used for the construction of a cocoon in which the insect pupates. However, the larvae of water midges, most Trichoptera and some Lepidoptera spin protective tubes or webs, catching nets, attachment pads, or walking guidelines during most of their larval life.

Silk composition has been studied extensively in the silkworm *Bombyx mori* and to lesser detail in a few other species of Holometabola (Sehnal and Sutherland, 2008). Labial glands of *B. mori* and other caterpillars are differentiated into a posterior, middle, and anterior section (Fig. 11.1). The posterior section (PSG) secretes large amounts of a high-molecular (200–500 kDa) heavy chain fibroin (H-fibroin), low-molecular (ca 25 kDa) light-chain fibroin (L-fibroin), and the chaperonin P25 (occurs in two differentially glycosylated forms of ca 27 and 31 kDa). An L-fibroin linkage with the H-fibroin C-terminus via a disulfide bond is indispensable for the secretion of both these proteins (Takei et al., 1987). Identification of homologous silk components in other Lepidoptera (reviewed by Fedič et al., 2002), including the relatively primitive suborder Yponomeutoidea (Yonemura and Sehnal, 2006), indicates that the silk filament composed of H-fibroin, L-fibroin, and P25 is a plesiomorphic feature of Lepidoptera. The composition has been modified in the evolutionarily advanced family Saturniidae whose silk filament is made of H-fibroin dimers without participation of either L-fibroin or P25 (Tamura et al., 1987).

Secretion from the posterior silk gland is pushed as a column into the middle silk gland section (MSG, Fig. 11.1), where it is consecutively enveloped by three layers of sericin secretions. The silk of *B. mori* harbors at least six sericin glycoproteins that range in size from 65 to 400 kDa (Gamo, 1982), contain a high proportion of serine (16–42%) and other hydrophilic amino acids (Komatsu, 1975; Gamo et al., 1977), and represent 20–30% of the cocoon proteins (Suzuki, 1977). The multitude of sericin proteins is derived from the genes *Ser1*, *Ser2*, and *Ser3*



Fig. 11.1 Right silk gland of the last instar larva of *Galleria mellonella*. PSG, posterior silk gland region where components (H-fibroin, L-fibroin, P25) of the silk filament are secreted. MSG, middle region producing sericins is composed of three functional parts: distal (loop anterior to PSG), central (straight central portion of MSG), and proximal (straight terminal MSG portion). ASG, anterior region where the liquid dope polymerizes into solid filament coated by several sericins. Note: relative proportions of PSG, MSG sections, and ASG, differ among Lepidoptera, and indicate differences in the amounts of corresponding secretions

that are differentially expressed in different MSG regions. Because of alternative splicing, the gene *Ser1* generates four and the gene *Ser2* two mRNAs; mutual ratios of mRNAs change during development (Michaille et al., 1986, 1990; Couble et al., 1987; Garel et al., 1997). Four sericin proteins were unequivocally identified as the products of sericin genes. *Ser1* generates a 150-kDa protein in the distal and a 400-kDa protein in the central MSG, *Ser2* produces a 250-kDa protein in the proximal region, and *Ser3* a 130-kDa protein in the central and proximal regions (Takasu et al., 2002, 2007). Allelic polymorphisms and possibly also different degrees of glycosylation add to the intricacies of sericin protein analysis; for example, proteins of 120 and 230 kDa (and not 250-kDa) were identified as *Ser2* products by Kludkiewicz et al. (2009).

The sericin layers do not mix when the fibroin column moves into the much narrower anterior silk gland (ASG) where proteins from the PSG solidify into a filament (Fig. 11.1). The cause of conversion of about 30% protein gel into the solid filament is not understood. It has been proposed that the non-covalent interaction of P25 with the H-fibroin N-terminus facilitates storage of the highly insoluble H-fibroin/L-fibroin heterodimer in the form of a jelly dope in the silk gland lumen (Tanaka et al., 1999). Interactions and possibly changes in the ratio of the three proteins seem to be involved in the filament formation which is based on weak molecular interactions between repetitive motifs that occupy 95% of the H-fibroin molecule (Sehnal and Žurovec, 2004). The spun-out silk filament contains H-fibroin,

L-fibroin, and P25 in a ratio of 6:6:1 (Inoue et al., 2000, 2004). The filaments – one from the right and the other from the left gland – move close to each other when the ASG fuses into a single silk gland outlet. Sericins from the most distal MSG region and thereby close to the fibroin column then seal the pair of filaments into a single fiber. The remaining sericins solidify after filament passage through the spinneret: some stick the fibers to substrate at the start of cocoon spinning and others glue the fibers in the cocoon wall.

The fibroins and sericins are the major but not the only components of the lepidopteran silks. Some of the small proteins identified in the silk (Kodrík, 1992) are possibly responsible for the silk's resistance to predators, molds, and microbes (Akai, 1997). This may be the role of seroins, which are derived from one gene in *G. mellonella* and from two genes in *B. mori* (Žurovec et al., 1998b; Žurovec and Sehnal, 2002; Nirmala et al., 2001b). Two other genes specifically expressed in the silk glands of caterpillars encode Kunitz- and Kazal-type protease inhibitors that are active on certain fungal and bacterial proteases (Nirmala et al., 2001a; Kludkiewicz et al., 2005).

11.1.2 The Ancient Technology of Silk Reeling

Large cocoons containing nutritious pupae have certainly attracted man since the stone age but their conversion to textiles required special technology that was developed in ancient China. According to a tale, the technology was discovered by coincidence when a Chinese princess dropped a cocoon into a cup of hot tea. She observed loosened fiber that could be pulled, dried, and reeled. This principle is still used today: cocoons are soaked in hot and slightly alkaline water for about 10 minutes to dissolve the sericins by which the cocoon is held together. Silk fibers loosened from the cocoon are picked up, about five are joined into a raw silk thread (fibers are glued together automatically by residual sticky sericins) that is slightly squeezed by passage through an eyelet, dried during this process, and reeled. The procedure of partial sericin solubilization that loosens the silk fiber from the cocoon is called degumming. The technology is used for the cocoons of about a dozen moth species (Peigler, 1993) among which the silkworm *B. mori* dominates. The oldest Chinese book on sericulture claims that silkworm rearing started in the Huang-Di empire, i.e. around 2,650 BC. *B. mori* was domesticated from the wild silkmoth *B. mandarina* and became fully dependent on human care without which it cannot survive.

Sericulture spread from China both to the East and West and became a very important industry. Since the caterpillars of *B. mori* feed exclusively on mulberry leaves, with a preference for the white mulberry, this plant also spread. The economic importance of sericulture lasted millennia, reaching a peak in the nineteenth and the first half of the twentieth century. With the invention of the parachute, silk became a strategic commodity that was replaced only by nylon which was discovered in USA during World War II. After a decline due to the production of cheap plastic fibers, silk production began to rise again in recent years. According to the FAO (Food and Agriculture Organization of the United Nations), worldwide silk

output rose from around 100,000 tonnes in 2000 to 150,000 tonnes in 2006, of which about 70% is produced in China, followed by Brazil, India, Thailand, and Viet Nam (http://www.naturalfibres2009.org/en/fibres/silk.html). Classical sericulture based on *B. mori* and several silkmoth species (Saturniidae) provides a living to millions. Attempts to use some more exotic species as silk producers are often motivated by the noble desire to help people in remote rural areas and at the same time contribute to nature conservation by promoting care of rare lepidopterans and their food plants. Cocoons obtained in the frame of such projects are typically processed into non-woven textiles or jewelry. The case of the Wild Silkworm Development Project of Indonesia (http://www.jog-ja.com/images/WildsilkEnglish01.pdf), which is based on the saturniids *Cricula trifenestrata* and the largest moth of the world, *Attacus atlas*, testifies that such an approach may be successful and very beneficial to the rural communities. A similar mission for Madagascar has been formulated as the CPALI project (Conservation through Poverty Alleviation; http://www.cpali.org) that "contributes to natural resource conservation by developing integrated, small enterprise systems that link the livelihoods of farm families and communities to the maintenance of natural ecosystems."

The quality of commercial silk has been modified for centuries by genetic selection of *B. mori*. More than 1,000 genotypic variants, which occurred either spontaneously or were induced by radiation or chemicals, have been registered (Fujii et al., 1998), but only a few of them are of value for practical sericulture. Modern techniques of molecular biology permit controlled modifications of silk properties, for example by the transfer of silk genes between species (Kobayashi et al., 2009). It is likely that genetic strains producing hybrid silks will be commercialized. Transgenesis based on silk gene modifications providing the silk filament with completely new features is also very promising. For example, Kojima et al. (2007) generated transgenic silkworms expressing fusion protein composed of H-fibroin and the enhanced green fluorescent protein EGFP. Cocoons spun by these silkworms emit green light (507 nm wavelength) when illuminated by light of about 480 nm. One can imagine silk clothes of unusual luster which change color on moving from day light to artificial illumination from glow lamps or fluorescent tubes.

11.2 Use of Natural Silk in Medicine

11.2.1 Silk Fibers

Silk has been used for textiles, bullet-proof vests, jewelry, various decorative materials, and also in medicine. Sutures from *B. mori* silk were indispensable in surgeries before synthetic materials became available. Sensitization to silk sutures, revealed by type-I allergic reactions like asthma and upregulated levels of specific IgEs, has been described in patients that underwent repeated surgery. The failure was explained, without real proof, by the presence of "residual" sericins in the silk thread (Altman et al., 2003). Since the poorly controlled degumming process removes various amounts of sericins, the surface properties of the sutures vary and it cannot be excluded that some sericin components, which remain on the sutures only

occasionally, are not fully compatible with mammalian tissues. However, Panilaitis et al. (2003) examined the direct inflammatory response under in vitro conditions in the cultures of murine RAW 264.7 macrophage cells and showed that native silk fibers coated with sericins as well as extracted soluble sericins were immunologically inert, while the insoluble fibroin particles induced significant TNF release. While sericins did not activate macrophages by themselves, they exerted a synergistic effect with bacterial lipopolysaccharide. The threat of allergic reactions calls for better characterization and subsequently better standardization of the silk products. The biocompatibility of native and recombinant sericins is discussed in Sections 2.2 and 3.2 of this chapter.

The biocompatibility of fibroin deprived of the sericin coating has been shown repeatedly in vitro as well as in vivo. Silk fibroin was used as a substratum for the culture of animal cells in place of collagen (Inouye et al., 1998). The mechanical properties of degummed silk fibers were improved by gelatin coating using tetrapeptide AsnAspGlyAla as a cross-linking agent; both the native and the gelatin-treated silk fibers were devoid of cytotoxicity (Liu et al., 2007). Subcutaneous implants of non-woven fibroin induced only a very mild graft response and provided a scaffold for the formation of reticular connective tissue (Dal Pra et al., 2005). Scaffolds made from porous fibroin and loaded with the bone morphogenetic protein-2 (BMP-2) induced osteogenic differentiation in human bone marrow stromal cells (Karageorgiou et al., 2006). Scaffolds loaded with BMP-2 and seeded with the marrow stromal cells supported bone ingrowth when implanted into critically sized cranial defects in mice (Karageorgiou et al., 2006) and femoral defects in nude rats (Kirker-Head et al., 2007).

The native spider silk also proved very useful in medicine. A group of German researchers developed a reeling machine that allows harvesting of long silk fibers spun by immobilized individuals of the spider species *Nephila clavipes* (Allmeling et al., 2006). In vitro assays revealed that the fiber promoted adherence, proliferation, and orientation of Schwann cells along the fiber axis (Allmeling et al., 2006). In a subsequent study, the spider silk fiber was used to bridge a 20-mm gap in the sciatic nerve of rats. Axonal in-growth and remyelinization were demonstrated. Successful regeneration was achieved with implants previously supplemented with Schwann cells and likewise with cell-free implants (Allmeling et al., 2008). It was concluded that spider silk is a viable guiding material for Schwann cell migration and proliferation as well as for axonal re-growth in a long-distance model for peripheral nerve regeneration.

Possible uses of the lepidopteran and spider silks in biomedicines have been reviewed and discussed by Hakimi et al. (2007).

11.2.2 Use of Sericin Products

Sericins washed off the silk fibers during the degumming process are often discarded, although efficient methods of their extraction from the waste water have been developed (e.g., Fabiani et al., 1996). Collected sericins are suitable for diverse

applications (Zhang, 2002; Padamwar and Pawar, 2004; Kundu et al., 2008). Their use in cosmetics has a long history and is still likely to expand because recent investigations showed that sericins possess moistening (Padamwar et al., 2005), UV-absorbent (Zhaorigetu et al., 2003a), antioxidant (Zhaorigetu et al., 2003b; Dash et al. 2008b), and to some extent also tumor-suppressing properties (Zhaorigetu et al., 2001, 2007). Since sericins were also reported to prevent wrinkles and other manifestations of skin ageing (Padamwar et al., 2005), their addition to skin creams and hair-care products is justified.

Several industrial applications of sericins have been proposed but probably none have been realized. For example, it was suggested that coating of air filters with the anti-oxidant and anti-microbial sericins would enhance their efficacy in polluted air cleaning (Sarovart et al., 2003). Sericins could also be used as biodegradable glues. Very high stickiness was found in the Sericin 2 proteins of *B. mori* that coat silk fiber produced at the start of cocoon spinning when firm attachment to a substrate requires exceptionally strong glue (Kludkiewicz et al., 2009). Biodegradable and immunotolerant sticky proteins would find both industrial and biomedical applications but the technology for their use still remains to be developed.

Biomedical sericin applications include remedies that are popular in East Asia and are based on experience. The whole silk or its sericin fraction is recommended as a treatment for ulcers, including protection against gastric lesions caused by excessive alcohol consumption (Li et al., 2008). Several pharmaceutical applications were proposed based on sericin activities in standard bioassays. For example, peroral application of sericin significantly reduced the incidence of colon adenomas in mice treated with a cancerogenic substance, presumably because sericin suppressed oxidative stress, cell proliferation, and nitric oxide production (Sasaki et al., 2000; Zhaorigetu et al., 2001). Topical applications of sericins protected mice against skin tumor induction by UVB-irradiation (Zhaorigetu et al., 2003a) or by 1,2-dimethylbenz-α-anthracene (Zhaorigetu et al., 2003b). A sericin extract from the cocoons of *Antheraea mylitta* was shown to inhibit apoptosis induced by UVB-radiation in human keratinocytes (Dash et al., 2008a).

Various other sericin applications in biomedicine have been proposed. Sericin was recommended as a vehicle for DNA delivery in gene therapy (Yanagihara et al., 2006), as an insulin conjugate increasing hormone half-life (Zhang et al., 2006), and in sulfated form as an anticoagulant (Tamada et al., 2004). However, the only commercial biomedical use of sericins today is in cell culture. The cell-growth-promoting activity of the sericin extract was first demonstrated by Minoura et al. (1995), who showed that cell attachment and growth on films made of silk required at least 90% sericin content. Terada et al. (2002) demonstrated with several cell lines and Ogawa et al. (2004) with the insulinoma cells of pancreatic islets that sericins not only support cell proliferation but may fully replace bovine serum products that were an indispensable medium ingredient for many cell types (Takahashi et al., 2003, 2005). The bovine products have several disadvantages, including possible transfer of pathogens. Sericin preparations for tissue culture media are produced and sold primarily in Japan (for example, http://www.wako-chem.co.jp). The positive effect of sericins on cell proliferation was recorded in various cell types (Sasaki

et al., 2005; Terada et al., 2005) but it is not general. Clinical applications are likely for pancreatic islet cells (Ogawa et al., 2004) and other cells that are propagated in vitro and then returned to the patient. The cryoprotective effect of sericins and their hydrolyzate increases their application potential for cell culture and conservation (Sasaki et al., 2005).

The mechanisms by which sericins stimulate cell proliferation and exert other biological effects are not known. The stimulation of human primary skin fibroblasts was traced to their better substrate attachment (Tsubouchi et al., 2005) but other factors must be of importance, too. Different cell types, for example the insulinoma cell line RIN-5F and the T-lymphocyte line CTLL-2, differ in their sensitivities to diverse sericin fractions. Terada et al. (2005) reported that sericin preparations ranging in size from 5 to 100 kDa were superior to a blend of 50–200-kDa sericins in promoting the CTLL-2 cells, whereas growth of skin fibroblasts was specifically stimulated by the 400-kDa sericin fraction (Tsubouchi et al., 2005). Poor standardization of current sericin products is probably the major obstacle to their use in clinical medicine.

The success of sericin application in tissue culture media spurred interest in their use as scaffolds for tissue reconstruction. Further advance in plastic and reconstructive surgery depends on novel biomaterials, which can be used as cell-seeded tissue constructs, in wound-healing therapies, and in skin regeneration. New therapeutic approaches to the reconstruction of lost body structures will probably employ stem cells stimulated to differentiate into the required tissue type on appropriate scaffolds. Films, fibers, and three-dimensional sponges made of silk proved to support proliferation and differentiation of various cell types (Altman et al., 2002, 2003). In particular, stem cell-based tissue engineering using 3D fibroin scaffolds has expanded the use of silk-based biomaterials for reconstructing a range of skeletal tissues like bone, ligament, and cartilage, as well as connective tissues like skin (Karageorgiou et al., 2006; Wang et al., 2006). Silk products are regarded as ideal materials for regenerative tissue engineering based on suitable stem cells. Soluble sericins can be polymerized into membranes and fibers (Zhang, 2002) or three-dimensional scaffolds of hydrogel consistence (Teramoto et al., 2005). The polymers are stable enough to provide time for tissue regeneration but due to their subsequent degradation do not interfere with the regenerated organ; this is an important requirement in tissue engineering. In other cases, sericin scaffolds can be used as a matrix for the fabrication of biomaterials that replace lost tissue permanently. Takeuchi et al. (2003, 2005) demonstrated that sericin coating of natural silk fiber facilitated apatite deposition from solution and could be employed in the fabrication of hybrid materials analogous to bone. Development of scaffolds based on sericins cross-linked to extra-cellular matrix proteins or to growth factors is another area of great practical potential. Finally, the anti-bacterial and cell-growth-stimulating effects of sericins might be extremely useful in applications such as coverage of complex and chronic wounds as well as a skin substitute in severe burns. The moisturizing effect is likely to promote wound healing and skin regeneration.

11.3 Recombinant Silk Products

There are two major reasons for the attempts to produce recombinant silk components: the high cost of natural silk and low yield and poor standardization of silk products other than the fibroin-based fibers. We are unable to mimic natural silks in their complexity, i.e. in the case of lepidopteran silk to produce fibers with core filaments made from H-fibroin, L-fibroin, and P25, sealed together by several sericins. Current efforts focus on the fibroin-type proteins that can make fibers or films, on proteins (usually sericin-type) that can be used in medicine and industry, and on minor silk components that possess anti-microbial properties. Insect genes are used only exceptionally. Most efforts are carried out with artificial genes that copy or mimic sequence motifs present in natural genes. Some of the "silk-like proteins" contain only a relatively short amino acid sequence resembling a silk protein. This is for example the case for a silk-elastinlike hydrogel proposed as a vehicle for cancer gene therapy (Megeed et al., 2004).

11.3.1 Filaments from Recombinant Silk-Type Proteins

Aligned H-fibroin molecules held together by hydrogen bridges and other weak molecular interactions are an essential component of the lepidopteran silk filaments. The interactions occur between repeated motifs of the amino acid sequence, for example the hexapeptide GAGAGY motif in the H-fibroin of *B. mori* (Mita et al., 1994), polyalanine motif in the *Antheraea* species (Sezutsu and Yukuhiro, 2000), and various other motifs of different length in other lepidopteran species (Sehnal and Žurovec, 2004). Proteins forming the filaments of spider silks contain similar repeats. The first step in the design of recombinant proteins for the fabrication of artificial filaments is therefore the choice of repeats and of their reiterations in the gene construct. The nature and arrangement of repeats must yield molecules that are soluble when stored as a dope and insoluble when aligned into a filament. Various vectors (bacteria, yeast, plants, and animals) have been used to express recombinant fibroin-type genes. Relatively large amounts of fibroin-type protein were obtained in some cases. However, controlled conversion of these proteins from solution to the solid filament proved difficult and has not been resolved to date.

Considerable success was achieved by the company Nexia that expressed fibroin-type protein derived from a spider sequence in transgenic goats (Lazaris et al., 2002). To this end, an artificial silk gene was attached to the promotor of the casein gene and inserted into the goat genome where it was specifically expressed in the mammary gland and accumulated in the milk. Technology for protein purification from the milk was established and filament formation was achieved by injecting the dope into a methanol bath. The filament was given the name "biosteel" to emphasize its strength. However, persistent production of a standard and reasonably priced product was not achieved and the operation was abandoned.

Several researchers were able to prepare films or to draw “filaments” from thick solutions of fibroin-like proteins but a consistent and reliable procedure of filament formation from a jelly dope has not been published. No one has been able to mimic natural spinning during which water withdrawal, pH change, exchange of ions, and enforced rheology contribute to the filaments formation (Knight et al., 2000; Jin and Kaplan, 2003). The spinning process causes permanent changes in protein conformation: while silk dope taken from a *B. mori* silk gland can be drawn into a fiber easily, dope reconstituted from cocoon filaments solubilized in 9-M LiBr yields at best a very inferior fiber (Holland et al., 2007).

Development of recombinant filaments to be used as scaffolds for tissue regeneration seems to be more successful than the production of strong textile fiber. Various modifications of native silk proteins have been tested. For example, scaffolds made from *B. mori* fibroin cross-linked by formic acid proved to be a suitable substrate for long-term cultures of epidermal keratinocytes and dermal fibroblasts that may form dermo-epithelial equivalents (Dal Pra et al., 2006). Proteins based on the consensus repeats of the major component of the dragline silk from the spider *Nephila flavipes* were combined with the ArgGlyAsp tripeptide to promote their interaction with integrins and thereby increase cell adhesion (Sofia et al., 2001; Chen et al., 2003). This modification stimulated differentiation of the tendon cells (Kardestuncer et al., 2006) and enhanced differentiation of human stem cells from the bone marrow, including osteogenesis (Bini et al., 2006).

11.3.2 Recombinant Sericin-Like Proteins

The structure of *B. mori* sericins (these proteins have not been studied in sufficient detail in any other species) shows considerable diversity (Table 11.1). All recombinant sericin-type proteins produced and tested so far were derived from the repeats of Sericin 1 and found to possess various properties of native sericins obtained by cocoon degumming. Tsujimoto et al. (2001) showed that dimers of the Sericin 1 repeat composed of 38 amino acid residues (Table 11.1) possessed cryoprotective activity. The dimer also prevented apoptosis in insect Sf9 cells exposed to culture medium which was deprived of the bovine serum products (Takahashi et al., 2003). Later studies showed that the SGGSSTYGYS portion of the 38-mer was sufficient to prevent cell death in the depleted medium (Takahashi et al., 2005). Aromatic residues seemed to be important because SGGSSTWGWS had the same

Table 11.1 Sericin repeats identified in *Bombyx mori*. Information on the sericin 1 repeats was taken from Garel et al. (1997), on the sericin 2 from Kludkiewicz et al. (2009), and on sericin 3 from Takasu et al. (2007)

Gene	Examples of most frequent repeats in encoded proteins
Sericin 1	SSTSGGTSTYGYSSRHRGGSV SSTGSSSNTDSSTKNAG
Sericin 2	SDKDTEKAKPNDRSP SHKDTEKVKPNDRSP
Sericin 3	SSRSQQAH SSSSKQAQ (plus an 86-amino acid repeat)

anti-necrotic activity as SGGSSTYGYS but the SGGSSTAGAS derivative was inactive. Huang et al. (2003) prepared recombinant proteins of 17.4, 31.9, and 46.5 kDa based on concatenations of the 38-residue repeat. The proteins were water soluble but when their concentrations increased during dialysis they formed β-sheets and self-assembled into fibers.

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Chapter 12
Biosensors on the Basis of Insect Olfaction

Sebastian Paczkowski, Bernhard Weißbecker, Michael J. Schöning, and Stefan Schütz

Abstract Biosensors utilize organic components like proteins, cells, or fragments of organisms within a technical device. By combining technical transducers with a biological sensory unit it is possible to reproduce the capability of natural sensors, which far exceed mere technical solutions in selectivity and sensitivity. For instance, by integrating the antenna of a Colorado potato beetle (*Leptinotarsa decemlineata*) in the gate of a field-effect transistor (BioFET), volatile trace compounds are directly detectable in the ppb range. A complex biochemical detection system in the antenna of the beetle serves as a filter for compounds that provide vital information about its environment. Another advantage of biologically inspired sensory solutions is the evolutionary tuning of the natural detection units towards compounds that are significantly correlated to ecological conditions. Nature has undergone millions of years of survival of the fittest to select compounds that serve organisms reliably as information to trace hosts, mating partners, enemies, or competitors. Because of the diversity of ecological interactions between animals all over the world the biosensor concept and biomimetic approaches can lead to numerous applications for tracking, surveillance and monitoring of environmental and industrial processes.

Keywords Volatile organic compounds (VOCs) · BioFET · Fire detection · Agricultural pests · Forest pests · *Post mortem* interval estimation · Meat spoilage detection

12.1 Definition and Basic Principles

Basically, a biosensor is a measuring device that traces chemical compounds, organisms, or physical measurands by spatially and functionally combining a biological

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component with a physical or chemical transducer (Lowe, 1985; Scheller et al., 1985). This definition suggests a wide field of possible applications, as it neither refers to any peculiar biological components nor does it describe any concrete physical measurands. Actually, the field of possible realizations of biosensor concepts is quite extensive.

The integrated biological component could, for instance, be an organic molecule, like an enzyme or an antibody. These biochemical structures perform distinctive tasks in organisms and therefore, interact with only a selected number of other chemical compounds that occur sometimes only in low concentrations. This selectivity can be utilized in a sensor concept that responds selectively and sensitively towards distinct compounds.

The bio-component can also be an organelle, cell, organ, or a complete organism. Here, the definition of a biosensor converges towards the definition of a bio-indicator that is defined as a plant or animal species whose appearance or absence in a habitat allows correlations to environmental conditions, like nutrition availability or air pollution. As in the case of a bio-indicator where a macroscopic measurand, for instance the number of lichen-species, is determined, also a biosensor on the basis of molecular bio-components has to display a measurable factor that is transduced into a physical measurand.

This leads to the second element of a biosensor: the so-called transducer. As the technological part of the sensor this compound is defined by its function and not by its mode of operation. Its task is to convert the specific biological/chemical interaction of the bio-component and an analyte into a physical/chemical response with a measurable output, generally electrical signals.

The first published application of a biosensor was a system to measure the concentration of glucose in the blood (Clark and Lyons, 1962). Here, the enzyme glucose oxidase was immobilized with a dialysis membrane on a platinum electrode. The enzyme metabolizes glucose to gluconolactone while the produced hydrogen is transformed to hydrogen peroxide under consumption of oxygen. This product is detected by an amperometric electrode. Alternatively, the consumption of oxygen can be recorded. The bio-component is in this case the dissolved glucose oxidase and the transducer, a platinum oxygen electrode. A permeable membrane keeps the solution around the electrode.

12.2 Types of Biosensors

Biosensors can be classified by the type of the used bio-component, the stage of development (generation), or the type of transducer.

12.2.1 Bio-Components

Enzymes (enzyme sensors) and antibodies (immuno sensors) are utilized as bio-components in biosensors where the substrate is detected by docking on a selective

receptor. One can differentiate between catalytic sensors and affinity sensors depending on whether the formation of a receptor/substrate complex yields a metabolic product or not.

A microbial sensor utilizes living cells, for instance coupled to an oxygen electrode that measures respiration processes of the cell by means of the oxygen uptake. This utilization of living cells or tissue opens new perspectives for biosensor applications. However, it also presents a technical challenge to cultivate functional cells on a transducer and to obtain a sufficient storage protocol for the biosensor (Rudolph and Reasor, 2001). Biological receptors or organisms (e.g., after genetic modification) can even be used for the detection of compounds that are not relevant to the organism itself but are of greater interest to humans. As an example, genetically engineered bacteria produce a fluorescent protein in the presence of uranium, which emits a green glow when irradiated by UV-light (Hillson et al., 2007). Another example is the application of complete organs, for instance insect antennae, which is described in detail in Section 12.5.

12.2.2 Generations of Biosensors

The above-mentioned glucose sensor of Clark and Lyons uses a membrane in order to immobilize enzymes on an electrode. This setup is also designated as a biosensor of the first generation (Scheller et al., 1985).

Enhancements to this technique are sensors where the biochemical receptors are directly bound to the surface of the transducer (2nd generation) or immobilized directly on an electronic control device, for instance a transistor (3rd generation). In particular, field-effect transistors (FETs) are convenient because of their high input impedance. The combination of a FET with a bio-component, for instance an insect antenna, is referred to as a BioFET (Schügerl, 1985; Schütz et al., 1997a).

12.2.3 Transducers

The most important types of transducers are electrochemical, optical, mass-sensitive, or thermal sensors (Rodriguez-Mozaz et al., 2006). An example of electrochemical transduction is the amperometric detection mentioned in Section 12.1, which is especially used for catalytic enzyme reactions.

Optical sensors can utilize the absorption, fluorescence, or bioluminescence of molecules and organisms as input.

One technical realization of mass-sensitive sensors is the piezoelectric sensor combining the surface of a quartz oscillator with an enzyme or antibody. When bio-components from the surroundings attach to its surface the total mass of the crystal changes and due to Sauerbrey's equation the oscillating frequency of the quartz also changes.

Thermal sensors detect the production or consumption of heat during biochemical reactions.

12.3 Applications of Biosensors

Conventional methods of instrumental chemical analysis, especially gas chromatography and mass spectrometry (GC-MS), require high laboratory costs. In this context, biosensors offer approaches for improved methods that allow real-time on-site analytics (Badihi-Mossberg et al. 2007).

To detect, for instance, a pesticide in farmland, standard methods require a labor-intensive extraction of the target compounds from the sample matrix. GC-MS analysis is then performed with a throughput of 20–40 samples maximum per day. Therefore, the manual labor for sample preparation and the operation of the instrumental setup is very cost-intensive, restricting these conventional methods to the survey of a limited number of samples.

A portable measuring device without extensive sample preparation is needed to establish convenient applications for practical on-site use (Rodriguez-Mozaz et al., 2006). A biosensor with an appropriate selectivity can detect compounds directly in their matrix without preceding extraction or purification. This will inter alia enable the recording of data in a high spatial and temporal resolution in order to follow a concentration gradient and thereby, localize the source of the pollution. Applications of biosensors are, for instance, the detection of pesticides, air pollutants such as formaldehyde or sulfur dioxide, polycyclic aromatic hydrocarbons (PAHs), herbicides, toxicants, or heavy metals (Badihi-Mossberg et al., 2007). The range of applications for biosensors is not restricted to the detection of chemical compounds, but also includes the sensitive and highly reliable detection of specific microorganisms (Turner et al., 2008). Examples are the detection of *Escherichia coli* in the meat-processing industry (Tokarskyy and Marshall, 2008) or in water (Theegala et al., 2008). However, biosensors provide less accurate quantifications of compounds in comparison with conventional techniques of trace analysis.

If there is an assessment of complex biological effects needed, like toxicity and mutagenicity of mixtures, the state of health of plants and animals as well as the quality of ecosystems, a biological integration of complex measurands is necessary which cannot be detected by compound-specific trace analysis. In this case, a technical combination of bio-indicator organisms to adapted transductors is necessary. An example is the detection of the biochemical oxygen demand (BOD) of water which displays the amount of oxygen demanded to catabolize the contained amount of organic matter. This complex measurand is an indicator for the rate of pollution by sewage and can be determined with different conventional methods that are not applicable for online measurement (Rodriguez-Mozaz et al., 2006). For this demanding task biosensor approaches are already commercially available (Liu and Mattiasson, 2002).

On the whole, it is to be expected that the role of biosensors in diverse fields of application will increase rapidly in the future. Some applications are already covered by commercially available systems and recent research highlights the large diversity of further possibilities.

12.4 Insect Olfaction as a Basis for Biosensors

The basic idea of a biosensor based on insect olfaction is to utilize the extremely high sensitivity and selectivity of olfactory receptors (ORs). The silk moth *Bombyx mori* is able to trace 1,000 molecules per second of the pheromone bombycol in one cubic centimeter of air (Kaissling and Priesner, 1970). This is equivalent to a 1-g sugar lump diluted within and distributed over the whole water volume of Lake Constance. Another advantage of an insect antenna is the evolutionary adaption of insect olfaction to the detection of complex environmental parameters. The black jewel beetle *Melanophila acuminata* can, for instance, detect the burnt scent of a single charred tree over a distance of several kilometers (Schütz et al., 1999).

Therefore, the combination of a highly specialized biochemically operating insect organ with a signal amplifying and processing electronic device can lead to a striking detection performance of organic trace compounds in the atmosphere. Such a biosensor system acquires its sensitivity and selectivity by means of a biochemical transduction pathway linking the occurrence of organic trace compounds to the depolarization of olfactory sensor neuron populations on the antenna. As the adaptation of the olfactory sense in insects to the distinctive demands for the single species has gone through millions of years of selection of the fittest, the resulting compounds can be regarded as marker compounds in the ecological interaction between the insect and its environment. Combining techniques of trace analysis, like gas chromatography/mass spectrometry with parallel electroantennographic detection based on insect antennae into one GC-MS/EAD setup (Weißbecker et al., 2004) allows the identification of single compounds that are selectively traceable by an insect via scent. As insect species appear in most ecosystems all over the world and are adapted to many, often very distinct olfaction-based interactions with their environment, the amount of potentially usable marker compounds for biosensors based on insect olfaction is extremely large. The applicability of these marker compounds for economic purposes implies that the outcome of biodiversity conservation is a considerable source of innovation.

12.4.1 The Biochemical Transduction Pathway in Insect Olfaction

Once an odor plume reaches the airspace around an insect, it enters nanopores in the cuticle of an olfactory sensillum. The cuticle covers the antenna in order to prevent mechanical damage and desiccation of the sensory neurons housed in the sensillum. These nanopores allow volatile organic compounds to diffuse into the sensillum lymph that surrounds the neurons inside the sensillum. So-called odorant binding proteins (OBPs), first discovered by Vogt and Riddiford as pheromone-binding proteins (PBPs) (Vogt and Riddiford, 1981), selectively bind and transport the mostly

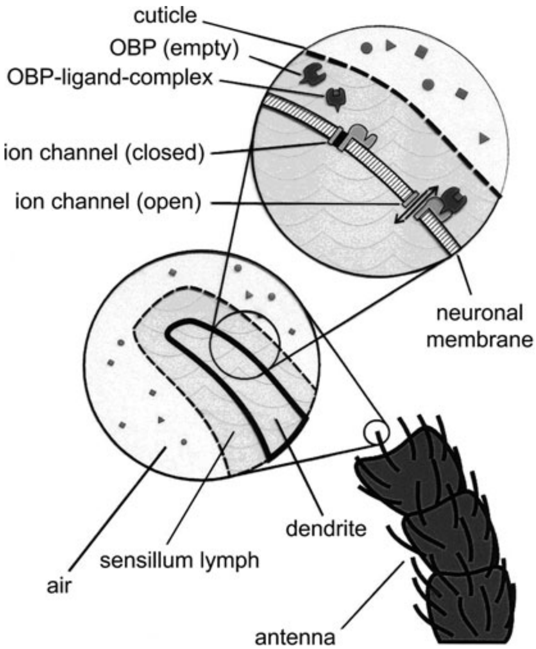


Fig. 12.1 The functional concept of insect olfaction. Embedded on the surface of the neuronal membrane inside a sensillum on the antenna are receptors that trigger ion influx by contact with an OBP-ligand complex

hydrophobic odor compounds through the hydrophilic sensillum lymph to the olfactory receptor contained in the neuron membrane (Fig. 12.1). It is proposed that due to the three-dimensional tertiary structure of these mono-chain proteins a binding cavity is formed in which functional groups of amino acids are exposed to interact with the peculiar structure and charge distribution of the odor molecule (Tegoni et al., 2004). When the OBP-ligand complex contacts its specific olfactory receptors on the neuron surface, a G-coupled protein cascade can be activated which leads to the opening of an ion channel allowing an influx of potassium ions that depolarizes the sensillum neuron. When the odor is present in a detectable concentration a sufficient number of cascades are activated to exceed the neuronal activation threshold and an action potential is elicited and further processed in the insect nervous system. Although intensive research has been conducted on understanding the biochemical interactions involved in insect olfaction the interactions are not as yet fully understood. For instance, the role of the OBPs and the ORs regarding the priority of selectiveness in binding the odor molecule is still a matter of discussion (Rützler and Zwiebel, 2005) and, as a second example, recently it has been found that the OR and the ion channel can form a spatial functional unit omitting the G-coupled protein transduction pathway (Wicher et al., 2008). However, the striking performance of insect olfaction in distinctively responding to ecologically relevant compounds has been verified by many studies and should lead to a wide range of artificial olfaction-based sensor applications in the future.

12.5 Application Layout: Biosensors on the Basis of Insect Antennae

The utilization of an intact insect antenna as a bio-component requires a mechanically and electrically stable junction while avoiding damage to the organ. Because of advantageous electrical and constructive possibilities the design of biosensors as BioFETs, meaning a hybrid device consisting of an insect antenna and a field-effect transistor (Fig. 12.2), has been established as particularly reliable (Schöning et al., 1998; Schroth et al., 2001).

In the case of the "whole-beetle" setup, the antenna of the Colorado potato beetle was directly coupled to the gate of the field-effect transistor via an electrolyte solution, whereas for the "isolated antenna" setup, it was removed from the beetle (here, one part of the antenna is connected via the electrolyte to the FET gate). A typical dose-response curve of this antenna-based BioFET is schematically depicted in Fig. 12.3: An air current, loaded with (Z)-3-hexen-1-ol, as an exemplary odor compound, is applied to the antenna. The biochemical cascade inside the antenna finally leads to the formation of an electrical potential drop across the receptor's cell membrane and thus, to a dipole potential over the whole antenna. The latter modulates the conductance of the FET channel between source and drain, inducing a distinct variation of the drain current I_D, which is dependent on the particular odor concentration.

The immanent miniaturization of the insect antenna as well as the miniaturization of the microelectronic part will allow manufacture of biosensor circuits of the size of a 1€ coin. Such sensor heads are deployable in portable biosensor systems to enable high-resolution data acquisition in-situ.

As distinctive examples four potential applications of biosensors on the basis of insect antennae are described below.

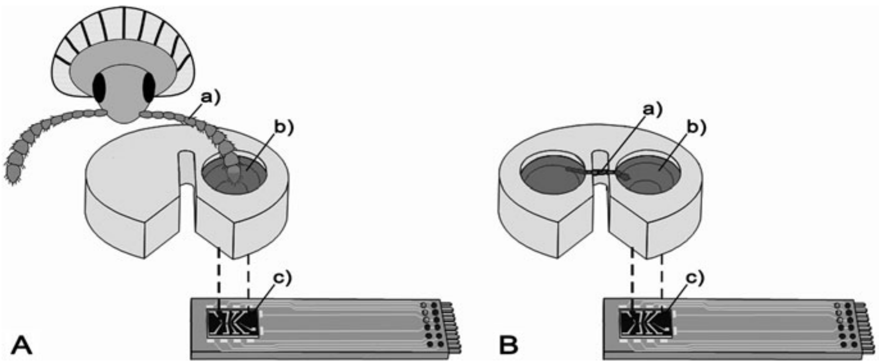


Fig. 12.2 BioFET hybrid consisting of an insect antenna and a field-effect transistor (FET) with intact Colorado potato beetle **(A)** and isolated antenna setup **(B)**, respectively. (a) Antenna, (b) electrolyte, and (c) field-effect transistor

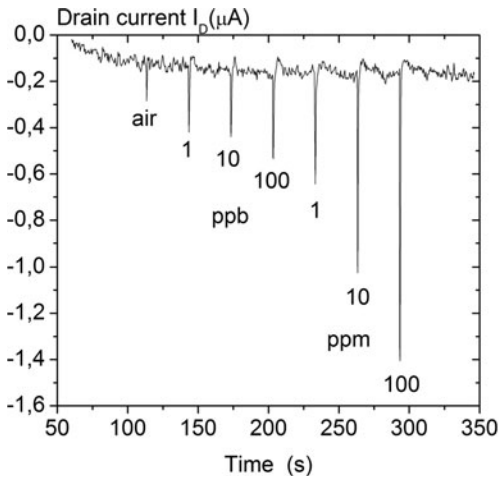


Fig. 12.3 Sensor response of the BioFET towards different concentrations of (Z)-3-hexen-1-ol in air. The change in the drain current I_D serves as a sensor signal

12.5.1 Fire Detection with Insect Antennae

Already at the beginning of the twentieth century there were reports from firemen of black beetles that flew in masses towards burning forests, hampering efforts to extinguish fires and even attacking firemen. Reports on this unusual pyrophilic behavior of the black jewel beetle *M. acuminata* included speculation that the beetle might be able to orientate towards a forest fire through olfactory means.

Further research on this beetle revealed that its antenna detects smoke components such as guajacol compounds very sensitively (Fig. 12.4).

These compounds originate from the pyrolysis of lignin, one of the basic constituents of wood. As the beetles breed in burned wood, they are adapted to the detection of wood fires like forest fires. Combustion of fossil wood (i.e., coal) can be detected by the beetles, too, as it also contains lignin.

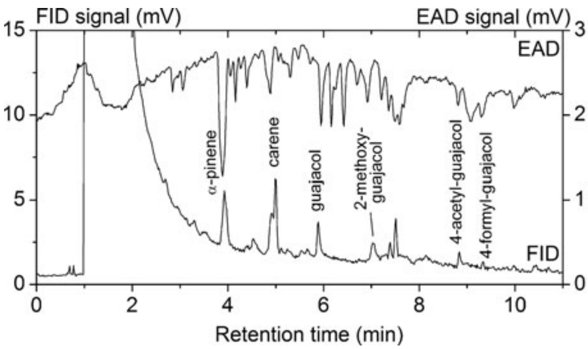


Fig. 12.4 Result of combined gas chromatography/flame ionization detection (GC-FID/EAD) utilizing the excised antenna fragment of *Melanophila acuminata*. Simultaneous peak formation allows the identification of electrophysiologically active compounds, in this case guajacol derivates and terpenes

Combustion products of diesel fuel or gasoline do not elicit comparable signals. This enables the design of a biosensor based on the antenna of *M. acuminata* to differentiate between frequently occurring background compounds, for example originating from traffic, and wood fire. Thus, false-alarms caused by engine exhausts can be avoided and a reliable fire early warning system can be based on the performance of the biosensor. Additionally, by distinguishing between different guajacol compounds the beetle is able to differentiate between types of fires, as the compounds originate from the variation of oxygen availability and fire temperature. As a fire warning device should be integrated into an emergency plan any additional information about the kind of fire will facilitate selection of appropriate response procedures.

Field experiments revealed that a fire comprising of only a few trees (5 m^3 wood) can be detected by a biosensor based on the antenna of *M. acuminata* over a 2-km distance and can be traced by the concentration gradient of smoke components and wind direction through a dense forest stand. Combining the high sensitivity and selectivity for marker compounds of fires with the identification of the kind of fire and the traceability of the source, a biosensor based on the antenna of *M. acuminata* is a powerful tool to be used as the first link in the fire-fighting chain.

12.5.2 Detection of Phytophagous Infestation in Agricultural Crops

The Colorado potato beetle *Leptinotarsa decemlineata* is one of the most important agricultural pests. Originally, the beetle occurred only on *Solanum rostratum* which is a solanaceous herb like the potato plant *Solanum tuberosum*. When American settlers intensified cultivation of potatoes the beetle infested these cultures as an additional source of nutrition. Since then the Colorado potato beetle has spread over the whole world and caused serious crop losses.

Investigations into the olfactory capabilities of *L. decemlineata* in search of a possible pest-management technique revealed that the beetle is able to differentiate between the feeding of conspecifics, infection of potato plants with the late blight disease (*Phytophthora infestans*), as well as the mechanical damage of potato plants via hail and agricultural machines by its olfaction. Thereby, it uses the fact that potato plants emit different scents when beetles feed on them compared to plants that are infected by fungi or that are mechanically damaged (Schütz et al. 1997b) and it uses different marker compounds for this discrimination.

These marker compounds are detected in very low concentrations and therefore, are recognized by the beetle even at long distances from the emission source. A marker compound for the cumulative type of damage is 2-phenylethanol, as its concentration in the air above a potato field allows correlation to the consumed leaf area (Schütz, 1997b). Only if cumulative damage surpasses an economic threshold does it make sense to apply control measures to avoid larger crop failure. Prophylactic chemical treatment can be avoided this way.

Infestation by the Colorado potato beetles is initially punctual and spreads as the population increases over the whole field. A well-directed control measure against infestation hotspots is possible by the detection of (Z)-3-hexen-1-ol, a marker compound correlated with the ongoing leaf damage. Therefore, monitoring of 2-phenylethanol and (Z)-3-hexen-1-ol by a biosensor based on the antenna of the Colorado potato beetle situated at a field margin or on a tractor enables the spatial assessment of cumulative and recent damage by taking wind direction and wind speed into account. This can result in on-site recommendations for efficient and economic pest management (Schütz and Weißbecker, 2003).

12.5.3 Assessment of Increased Infestation Disposition for Insect Forest Pests

The change to alternative energy sources such as renewable primary products (e.g., timber) has led to a focus on intensified utilization of our forests. As the value of timber increases its harvesting implies a profit gain and this consequently requires effective management systems. When land usage is intensified in forest stands possible risks have to be considered, for example nutrient loss, drought stress, or sunburn. These factors primarily lead to a decrease of accrescence and debilitation of the trees. A secondary effect is, for instance, the attraction of insects by the modified scent of debilitated trees. As the defense capability of such trees is lowered the survival rate of the insect's offspring rises. This leads to an increase in the pest's population, which is then able to infest intact trees, as well. Such complex ecological interactions can cause high economic losses. Therefore, a biosensor system on the basis of a pest species antenna to detect debilitated trees would enable early retaliatory actions.

The blue pine jewel beetle *Phaenops cyanea* is able to distinguish between short but vitality-decreasing drought stress, for instance on pine, and stress inflicted by fungi infestation or mechanical damage, for example after storms. It utilizes the fact that pines emit different scents when under drought and when stressed by fungi or mechanical damage (Schütz et al., 2004) and it is able to recognize the different marker compounds. These marker compounds are even detected in the ppb range (Schroth et al., 2001) and therefore, can be traced over long distances to damaged pine stands. Consequently, the antenna of the blue pine jewel beetle has the required sensitivity and selectivity when integrated into a biosensor setup. In order to detect long-term drought stress the antenna of the longhorn beetle *Monochamus galloprovincialis*, which is able to recognize selectively cyclic and oxidized monoterpenes emitted by trees under such stress situations, can be utilized (Weißbecker et al., 2006). For a practically relevant determination of whether a tree stand is damaged seriously enough to expect an infestation by *P. cyanea*, quantitative information correlated to the degree of damage is required. The marker compounds

(Z)-3-hexen-1-ol and nonanal are correlated with the degree of cell-decomposition in the bast of the trees. The detection of these marker compounds in a pine stand enables one to conclude the degree of damage of the assimilate-conducting tissue and thereby, the predisposition for an infestation by *P. cyanea* (Schütz et al., 2004).

12.5.4 Post Mortem Interval (PMI) Estimation in Legal Medicine

In legal medicine the colonization of corpses by necrophagous insects is used to estimate the time elapsed since death. Different insect species colonize a dead vertebrate in waves, each associated with distinct stages of decay. Studies on a vertebrate's volatile profile in a certain state of decay have shown that there is a high variation in the qualitative scent composition (Statheropoulos et al., 2005, 2007; Dekeirsschieter et al., 2009). To investigate any constantly occurring trace compounds during vertebrate decay on the basis of trace analysis would probably exceed the realistic limits of manual data acquisition. Insect species can be utilized in *post mortem* interval estimation to avoid significant expense in regards to time and money as they have already overcome the problem of volatile pattern variation. For instance, in a GC-MS/EAD setup the odor compound selection of several insects associated with early and later stages of decay are analyzed to reveal marker compounds that are typically emitted in these stages. According to the odor selection there are basically two types of carrion-colonizing insects, a general type that occurs during most of the decay period and a specialized type that occurs only at distinct stages of decomposition. Both types are valuable when a significant marker compound dynamics leading to a *post mortem* interval estimation is required. The former type can be used to develop a biosensor to indicate and locate the presence of corpses, while the latter type can provide quantitative patterns distinct for earlier or later stages of decay. The comparative relative quantity of compounds can lead to a dynamic marker compound pattern as a common core of the scent of decay. A suitable species for discovering such marker compound patterns may be, for instance, *Calliphora vicina*, a bluebottle blowfly species, as it occurs shortly after death until advanced decay (Smith, 1986). Odor compounds filtered by this fly species are—for example sulfur compounds like dimethyl-trisulfide (Stensmyr et al., 2002)—general compounds emitted during various metabolic activities. By utilizing the antennae of this fly species in a biosensor setup it is possible not only to estimate the *post mortem* time, but also to trace corpses or victims of natural disasters, for example after earthquakes. Insect antennae of specialized types of necrophagous insects should be selective for the identification of marker compounds characterizing certain stages of decay. For instance, there should be an increase of compounds associated with fermentation during the bloated stage and an increase in compounds associated with aerobic metabolic activity during the successive stage where the skin ruptures and the inner matter comes into contact with air. Combining the odor selection of both types of necrophagous insect antennae could lead to a biosensor setup that enables the estimation of *post mortem* interval as well as the tracing of corpses.

12.6 Biomimetic Approaches to Sensors on the Basis of Insect Olfaction

Analyzing odors emitted due to the metabolic activity of organisms adapted to an ecological context usually results in a very high qualitative and quantitative variation of the odor-constituting compounds. Despite this high variation insects are able to orientate towards the source of the scents by olfactory means. Taking into account recent knowledge of the biochemical chain of odor compound detection this implies that insects filter marker compounds from the whole scent emitted from the desired source, despite variation factors, such as environmental conditions (e.g., temperature) or metabolic activity of other organisms (e.g., microorganisms). Such marker compounds significantly display distinct conditions of dynamic ecological processes. However, prior to transferring them to the databases of functional sensor systems for monitoring these processes, limitations in the information content of the marker compounds as well as technical limitations have to be taken into account. Limitations in the information content of the marker compounds have to be considered according to the requirements of the technical sensor solution. A marker compound that significantly defines a natural process is not necessarily conferrable to an artificial industrial process or a processed industrial product. For instance, using the detection of a thermal combustion product as a marker for smoldering wood might not be significant for smoldering wood that has been impregnated. Regarding the emission rate of compounds, the infestation of natural wood by fungi should lead to quantitatively different emission progress compared to infestation of chemically treated wood. Although nature has provided an approach towards the solution of such cases, for example early fire detection utilizing the odor filtering abilities of *M. acuminata*, adaptation to alterations of natural processes is required.

Considering the performance of biosensors on the basis of insect antennae it is not possible yet to mimic its high sensitivity and selectivity in detecting single compounds by an artificial solely technical device. Anyway, progress in gas sensor applications provides devices with certain advantages in comparison to biosensors. The major drawback of the latter is the short life-time of the antenna which restricts its efficient usage. Although the requirements of running a biosensor are not high, there is a requirement for personnel to operate the systems.

Thus, biosensors are more useful for on-site screening or tracking purposes than for long-term monitoring purposes. In contrast solid-state sensors like semiconductor gas sensors have a lifetime of approximately 1 year, which makes them good candidates for low-cost monitoring applications in industrial processes. As they do not possess the selectivity and sensitivity of biosensors their applicability is restricted concerning the concentration of the compounds to be traced and the level of identification, which is rather compound groups, like aldehydes or amines as opposed to single compounds. Applications of semiconductor gas sensors include, for instance, monitoring the shelf-life of fish (Nicolay, 2006), monitoring of wheat for fungal contamination (Presicce, 2006), detecting different mold species in buildings (Kuske, 2006), and smoldering fires in coal storage units (Schütz et al., 1999) or the discrimination of toasted or untoasted bread (Bock et al., 1997). To combine this

technique with the concept of marker compounds is quite challenging, as the sensor has to be modified to respond selectively and to gate out any other compound reactions. This can be achieved with semiconductor gas sensor arrays where the combined reactions of all sensors in the array lead to a compound-specific pattern. Moreover, sensor filters, sensor surface modifications, temperature cycles, algorithm analysis, or artificial neuronal networks (ANN) serve to improve the sensors performance. As the efficient traceability of marker compounds is a valuable aim, the potential for enhancements in this field of technological research is high in order to successfully utilize the blue prints from nature. For instance, as outlined above, the amount of 2-phenylethanol emitted by potato plants or cyclic and oxidized monoterpenes emitted by trees under drought stress can be used to determine the ecological status of the field or the tree. The following examples describe further possible applications of biomimetic semiconductor gas sensor systems in industry.

12.6.1 Detection of Meat Spoilage

In our modern food industry quality control is the most important link between producer and customer. Trust is a strong marketing factor, but due to several scandals in recent times it became obvious that the producer is not always able to guarantee a minimum quality. Especially in the meat industry this is still a problem. Therefore, there is a strong need for innovation in this field.

Generally, any cells including muscle cells contain fat due to phospholipid membranes. Fatty acids, like linolic acid or arachidonic acid are typically abundant in membrane lipids and can be degraded to volatile aliphatic alcohols and aldehydes. Intra-muscular fat consists of fat cells inside the muscular tissue. Because of a permanent turnover in all tissues, the composition of nutrition has a strong influence on the composition of the triacylglycerides. The second important source of meat volatiles are proteins. The amino acid concentration of muscle tissue shows variations between species of animals (Branscheid et al., 2007). Amino acid chains in meat tissue are mainly assembled to form the basic functional unit of the myosin-actin complex that enables neuron-induced movement.

As the basic function of this complex is the same in all vertebrates, it is supposed that its biochemical properties and therefore, the amounts of different amino acids are the same, as well. Equal to protein-induced lipid degradation processes, meat tissue contains proteolytic enzymes that cleave muscle proteins *post mortem*. The main changes take place by fragmentation of myofibrils through the z-disk, degradation of desmin, nebulin, and titin and the appearance of two polypeptides with a molecular mass of 95 and 30 kDa. These phenomena are most likely induced by calpains and cathepsins. Putrescine, cadaverine, tyramine, and histamine are products of bacterial metabolism and *E. coli* play an important role in the formation of histamine and cadaverine (Durlu-Özkaya et al., 2001). The variability of biogenic amines occurring during fermentation processes of meat is very high and depends on the microorganisms growing on the tissue. As these amines are not volatile under moderate temperature conditions a further degradation is needed

to get a protein-induced flavor. Sulfur compounds are volatiles usually emitted by decaying meat (Mayr et al., 2003), fermented meat (Barbieri et al., 1992), and also cooked meat (Shahidi, 1998). It is most likely that these volatiles have their origin in methionine and cysteine, which are the major sulfur-containing compounds in meat tissue.

There has been some effort to correlate the volatiles emitted by decaying meat to its grade of spoilage (Stutz et al., 1991; Winquist et al., 1993; Nicolay, 2006; Mayr et al., 2003). These efforts resulted in sensor concepts disregarding the high diversity of the scent of decay, which limits their application range (Winquist et al., 1993; Nicolay, 2006).

As *C. vicina* is an organism associated with the colonization of vertebrate matter shortly after death it should be able to detect compounds emitted in the early phase of decomposition. These compounds can be regarded as marker compounds for the early spoilage of vertebrate tissue. Thus, by adapting a semiconductor gas sensor system to the dynamic marker compound pattern of aging meat it is possible to estimate the freshness of meat. Such a sensor system could be applied along the whole chain of custody from the slaughterhouse to the consumer. The most important restriction of such a sensor system is the detection threshold for the marker compounds as the emission rates in the early stages of spoilage are usually low.

12.6.2 Early Fire Warning System in Wood Flake Driers

The pyrophilic beetle *M. acuminata* has already been described above including its ability to detect fire over a long distance. Once it has reached the freshly burned area the female beetle oviposits on stems that were not completely destroyed by the fire. The hatching larvae feed on the cambium and later on the intact wood tissue. To be able to find an appropriate place for oviposition, the beetle has to be able to distinguish between different stages of heated or burned wood tissue. This ability can result in a marker compound pattern that significantly correlates the emission of compounds during wood heating with the temperature of the wood. This pattern, when detected by a semiconductor gas sensor system, can lead to fire warning applications that can detect wood fires before they ignite. Such applications can be adapted to, for instance, wood flake driers in the wood processing industry. Reducing the water content of flakes in order to glue and press them to boards requires a large energy input. As the drying process proceeds more efficiently at higher temperatures it would be more efficient to dry the flakes just under the threshold of self-ignition. Under production conditions the temperature is usually lowered to a level that ensures fire safety by monitoring the air temperature and installing spark extinguishing systems. Monitoring the marker compounds for heated wood just before the threshold of self-ignition would enable the temperature to be increased in wood flake driers without increasing the fire risk. This has the advantages of reducing energy consumption, the loss of material, and damage due to fire. Such systems can also be installed in biomass storage units, for

example self-ignition of saw dust that is stored in silos as a renewable energy source is one of the problems faced by modern energy plants. Applications for wooden constructions, like warehouses, hotels, or private residential buildings are possible, although the relatively high energy consumption of semiconductor gas sensors limits commercial viability in the private market.

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Chapter 13
Insect-Inspired Technologies: Insects as a Source for Biomimetics

Stanislav N. Gorb

Abstract The understanding of functional principles of insect materials, structures, sensors, actuators, locomotion, control systems, and behavior is of major scientific interest. On the other hand, this basic knowledge is also highly relevant for technical applications. One of the greatest challenges for today's engineering science is miniaturization. Insects have solved many problems correlated with extremely small size, during their evolution. Zoologists, entomologists, morphologists, and neurobiologists have collected a huge amount of information about the structure and function of such living micro-mechanical systems. This information can be utilized to mimic them for industrial applications. Insect solutions may be applied in the following main technology areas: (1) materials science and technology, (2) surface science, (3) science of adhesives, (4) optics, (5) photonics, (6) sensorics, and (7) robotics. A few selected examples are discussed in this chapter, but with over one million described species as a source for inspiration, one can expect many more ideas from entomological science for biomimetics.

Keywords Biomimetics · Biologically-inspired technology · Entomology · Insects · Surfaces · Biomechanics

13.1 Introduction

Throughout evolution, insects have solved a variety of technical problems and have evolved an immense variety of shapes and structures. Although often intricate and fragile, they can nonetheless deal with extreme mechanical loads. Many functional solutions are based on a variety of ingenious structural solutions. The rich sensory equipment of insects including complex eyes, various chemoreceptors, mechanoreceptors, etc. taken together with a compact brain, reveals interesting motion control patterns and remarkable behavioral features.

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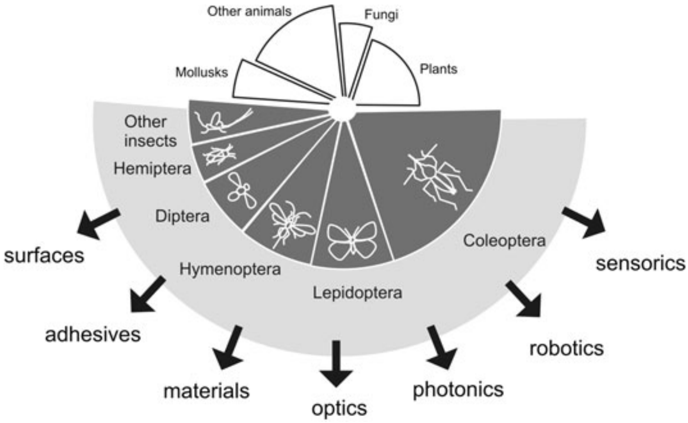


Fig. 13.1 Diagram of insect diversity as a source for biomimetic ideas in various areas of technology

The understanding of functional principles of insect materials, structures, sensors, actuators, locomotion, control systems, and behavior is of major scientific interest, enhancing our knowledge on their function and biological role. On the other hand, this knowledge is also highly relevant for technical applications. One of the greatest challenges for today's engineering science is miniaturization. Insects and other animals have solved many problems dealing with extremely small size, during their evolution. Zoologists, entomologists, morphologists, and neurobiologists have collected a huge amount of information about the structure and function of such living micro-mechanical systems. This information can be utilized to mimic them for diverse technical applications.

Insect solutions may be applied in the following main technology areas: (1) materials science and technology, (2) surface science, (3) science of adhesives, (4) optics, (5) photonics, (6) sensorics, and (7) robotics (Fig. 13.1). Possible innovations may also appear on the boundary between insect science and the areas listed above. Only a few selected examples are discussed in this chapter, but with over one million described species as a source for inspiration (about one half of eukaryotic organisms), one can expect many more ideas from entomology for biomimetics.

13.2 Materials

The insect exoskeleton is called a cuticle. Being an interface between a living animal and the environment, the cuticle of an insect serves many functions. (1) It limits the dimensions of an exoskeleton and is a basis for muscle insertion (mechanical function and function of locomotion). (2) It is an important element in organism defense against a variety of external factors such as mechanical stress, dry, wet,

cold or hot environments. (3) It takes part in the transport of diverse epidermal secretions, and serves as a chemical reservoir for the storage of metabolic waste products. (4) A variety of cuticular structures are parts of mechano- and chemoreceptors. (5) The cuticle, its coloration pattern, and chemical components are important for thermoregulation, and are often involved in diverse communication systems. (6) Specialized cuticular protuberances may serve a variety of functions, such as oxygen retention, food grinding, body cleaning (grooming), etc.

Cuticle is a layered composite material (Fig. 13.2), which consists of two principal components: chitin and protein. Chitin is a structural polymer, found in insects in crystalline form. The structure of chitin molecules and their arrangement are well studied, but the structure of chitin–protein assemblages still remains unknown. There are three morphs of chitin differing in the molecule packing and polarities of adjacent chains: α-, β-, and γ-chitin (Rudall, 1963). The arrangement of molecules usually varies within the different morphs of chitin. All three morphs of chitin are classified as visco-elastic polymers (Hepburn and Chandler, 1978). Chitin crystals always occur in insects as bundles of microfibrils with a diameter of 25–30 Å. Microfibrils are always associated with protein in a chitin–protein complex. Chitin-bearing cuticular microfibrils have a complex pattern of orientation in the three-dimensional space of the cuticle.

Arthrodial and caterpillar-like cuticles are materials of great flexibility, great extensibility, and reasonable strength. The interaction between chitin fiber and protein matrix is very loose, as has been demonstrated in deformation and fractographic studies. Under tension, these cuticles are visco-plastic and show so-called "necking," as do the steel and other metallic specimens under the same conditions (Hepburn and Chandler, 1976). However, there is remarkable variation regarding the degree to which such cuticles can be extended (Vincent and Wood, 1972; Vincent

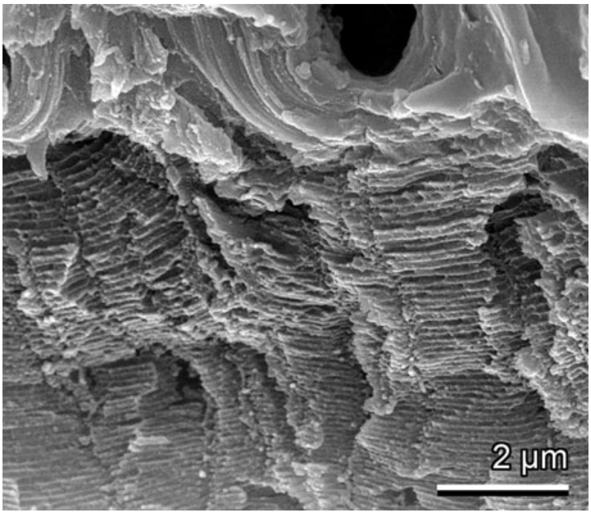


Fig. 13.2 Fracture through the cuticle of the rear side of the head in the dragonfly *Aeshna mixta*. Note the layered structure of the exocuticle

and Wegst, 2004). There are two types of membrane design found in insects. Highly extensible membranes that can extend more than 1000% of their length are found in the locust abdomen (Vincent, 1981). This cuticle is highly specialized at the molecular level due to its protein composition (Hackman, 1975; Hackman and Goldberg, 1987). The explanation for such extreme extensional ability is the fact that chitin fibers are arranged normally along the longitudinal axis of the animal, and one can presume that the protein matrix is not completely bound to the chitin fraction. The second type of membranous cuticle is folding laminated cuticle with a somewhat lower degree of extension. This cuticle, such as that found in the abdominal membranes of the tsetse fly, *Glossina morsitans*, is often armored with parallel rows of microtrichia (Hackman and Goldberg, 1987).

Resilin is a protein whose molecular structure consists of a three-dimensional network of chains; they are thermally agitated, randomly kinked, and fixed within a network of only a few stable cross-links (Andersen, 1963, 1964, 1966). These covalent cross-links are fluorescent amino-acids derived from tyrosine (Andersen and Weis-Fogh, 1964). The amino acid composition of resilin from different species and body parts is quite similar (Hepburn, 1985). Resilin was initially described in the prealar arm and wing-hinge ligaments of the locust, *Schistocerca gregaria*, and in the pleuro-subalar muscle tendon of the dragonfly, *Aeshna grandis*. These preparations showed recoverable mechanical deformability, so that the resilin-containing cuticle was called rubber-like cuticle, in contrast to the "solid" cuticle. Resilin can be reversibly distended over 200% of its initial length. Its principal physical properties are its long-range elasticity, and lack of either creep or stress relaxation (Weis-Fogh, 1960, 1961; Andersen and Weis-Fogh, 1964). The resilin-containing structures work as mechanical springs under tension and compression (Rothshild et al., 1975; Frazier et al., 1999; Gorb, 2004). Resilin has been revealed in different areas of insect wings (Gorb, 1999; Haas et al., 2000, 2000a), where it presumably prevents material fatigue in folds and promotes wing profile adaptation to aerodynamic situations in flight.

Insect cuticle demonstrates, in different functional systems, a gradient of material properties, which may range from very stiff areas of condyli of joints to membranous areas between leg segments. These gradients depend on the fiber density, fiber orientation, polymerization degree of the matrix, and thickness of single layers.

Meanwhile composite materials are spread widely in various areas of technology. However, fiber size remains in the micrometer range and fibers normally have random or preferable orientation within the matrix. Insect cuticle may inspire materials scientists with its helicoidal arrangement of nanofibers in successive layers and with its gradient-like materials properties.

In the last years, many efforts have been undertaken to artificially synthesize the rubber-like material protein of the cuticle (Elvin et al., 2005). There are many areas of potential applications for such a material ranging from medical rubbers to technical sealing systems.

13.3 Surfaces

Insect surface structures may serve many different functions, such as air retention, food grinding, body cleaning, etc. (Fig. 13.3). Scanning Electron Microscope images of some examples of such surfaces are shown in Fig. 13.4.

Because of the structural and chemical complexity of insect surfaces, exact working mechanisms have been studied for only a few systems. In relation to the broad diversity of functions of insect surfaces, inspirations from entomology are currently in focus in a broad range of research topics in engineering sciences: adhesion, friction, wear, lubrication, filtering, sensorics, wetting phenomena, self-cleaning, antifouling, thermoregulation, optics, etc. Since insect surfaces are multifunctional, it makes them even more interesting from the point-of-view of biomimetics.

One of the challenges in designing moving parts of micro-electro-mechanical devices (MEMS) is the fabrication of joints allowing precise motion of parts about one rotational axis or multiple axes. One problem is the high friction, stiction, and wear rate of joints (Scherge et al., 1999; Komvopoulos, 2003). Wear of the interacting surfaces is a consequence of friction, affecting the material's contact points by becoming deformed or being torn away. Friction and wear are strongly correlated processes by which the points of the surfaces in contact change their topography continuously. Capillary adhesion, due to the presence of a water layer in contact, can account for a great part of the measured friction or lead to the stiction between a contact pair (Scherge et al., 1999). These are critical issues limiting the operational lifetime and negatively influencing the technological potential of MEMS. Conventional methods of lubrication cannot always be used, especially in devices with medical applications. Friction reduction in some man-made mechanical systems is based on the different hardness of elements in contact (Miyoshi, 2001; Li et al., 2004), on the use of hydrophobic surfaces, and on applying surface texture, which minimizes the real contact area between two solid surfaces. Ideas from

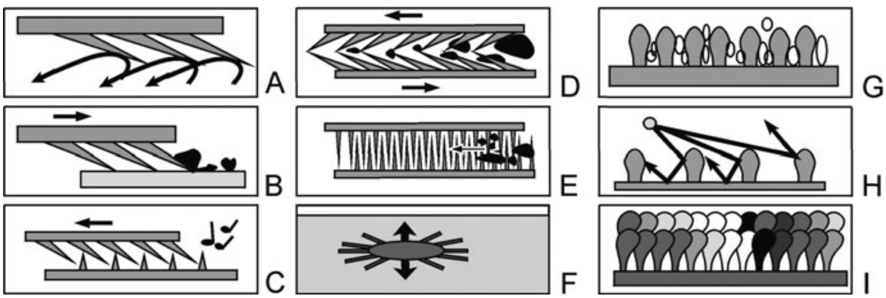


Fig. 13.3 Diagram of functions of cuticular microstructures in insects. **a** Aerodynamically active surfaces. **b** Body cleaning. **c** Sound generation. **d** Food grinding. **e** Filtration devices. **f** Hydrodynamically active surfaces. **g** Air retention. **h** Thermoregulation. **i** Body coloration pattern (Gorb, 2001)

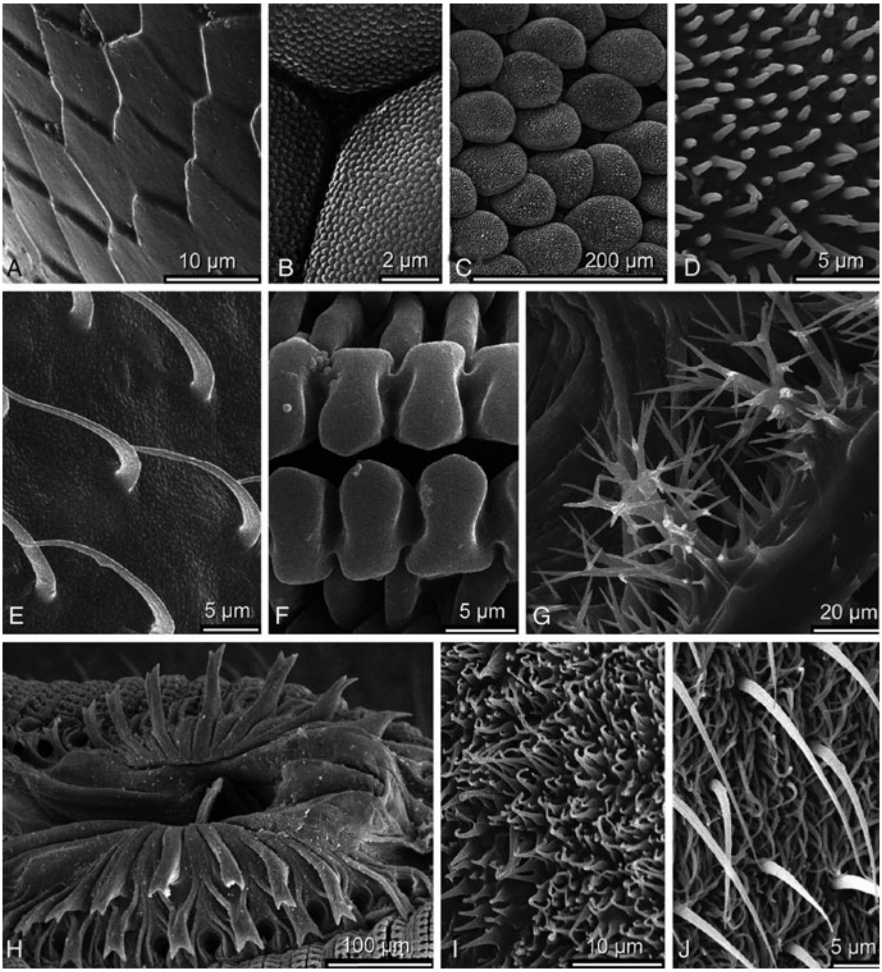


Fig. 13.4 Functional diversity of non-innervated cuticular protuberances in insects. **a** Unspecialized polygonal surface on the tarsus of the scarabaeid beetle, *Melolontha melolontha*. **b** Ommatidia surface in the calliphorid fly, *Calliphora vicina*. **c** Scales on the dorsal surface of the elytron in the scarabaeid beetle, *Hoplia* sp. **d** Same, the surface of a single scale. **e** Wing surface in the bibionid dipteran, *Bibio ferruginatus*. **f** "Pseudotrachea" of the labellum in *C. vicina*. **g** Filter system of the spiracle in the tenebrionid beetle, *Tenebrio molitor*. **h** Prestomal teeth in *C. vicina*. **i** Plastron in the nepid bug, *Ranatra linearis*. **j** Air retaining hair coverage in the water strider, *Gerris lacustris*

studying surface properties of insect joints, which have stiffer outer layers located on softer ones, specialized microstructure on the contact pair (Fig. 13.5), and particular fiber orientation in the exocuticle, might represent an interesting set of principles towards a solution (Barbakadse et al., 2006; Perez Goodwyn and Gorb, 2004).

Insects move on land, in the air, in soil, and in water. The resistance against motion mediated by surrounding media and by mechanical contact with various

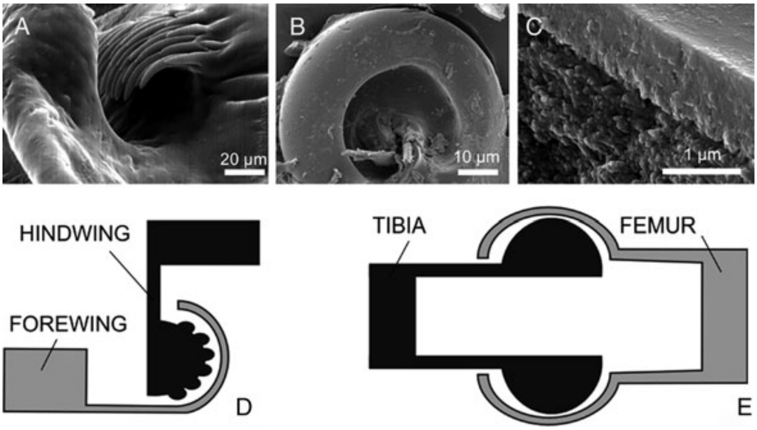


Fig. 13.5 Examples of microjoints in insects. **a** Lateral view of the wing double wave locking mechanism in the bug *Coreus marginatus* (*forewing part*). This joint provides interlocking between both wings on the same side of the body in the anterior direction allowing them to slide in the medial and lateral directions (Perez Goodwyn and Gorb, 2004). **b** Medial aspect of the femoro-tibial joint (*femoral part*) of the leg in the beetle *Melolontha melolontha*. **c** Fracture of the material of the joint in the beetle *M. melolontha*. **d** Diagram of the wing locking mechanism shown in **a**. **e** Diagram of the femoro-tibial joint shown in **b** and **c**. Constructional and mechanical principles found in such joints can be used to design joints in technical actuators

substrates was an evolutionary factor that contributed to the appearance of many surfaces adapted to reducing such resistance. A living motion system becomes optimized when it is capable of minimizing friction at one end of the system while maximizing it at the other end (Radhakrishnan, 1998). In other words, insects, like any other organism, need a combination of maximum friction required for acceleration, deceleration and maneuvering, and minimum friction in joints for economic energy expenditure. Adhesion and mechanical interlocking phenomena can also positively or negatively contribute to the functionality of such a system.

In insect joints working under lower loading forces, but much higher frequencies than vertebrate joints (Wootton and Newman, 1979; Gronenberg, 1996), the joint surfaces are usually smooth or present a combination of wavy and smooth counterparts (Fig. 13.5). Underlying tissues are penetrated with canals, which are presumably responsible for delivering lubricants to the contact area. The specialized surface structures have been shown to confer friction-reducing properties in insect joints (Perez Goodwyn and Gorb, 2004). Technical developments of such contact pairs are currently under way.

Small bristles, scales, and microtrichia of the wings of flying insects (Bocharova-Messner and Dmitriev, 1984) presumably may generate microturbulences around such structures in flight due to building a kind of lubricating layer of air between an air flow and the insect's surface. This could possibly decrease friction during high-speed flight.

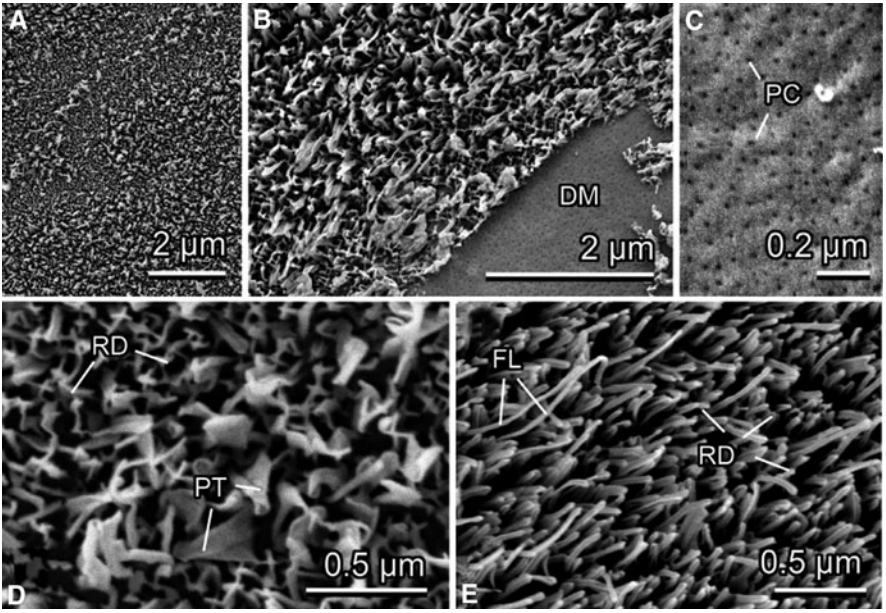


Fig. 13.6 Dorsal wing surface of the dragonfly *Calopteryx splendens*, young mature male. **a**, **d**, **e** surface of wing membrane covered with crystalline wax. **b, c** wing membrane surface with damage (*scratches*) in wax crystalline layer. *DM*, damage/scratch; *EX*, exocuticle; *FL*, wax filaments; *PC*, porous channels of cuticle; *PT*, wax platelets; *RD*, wax rods; *WC*, wax crystals (Gorb et al., 2009)

Some insect surfaces are covered by a superhydrophobic (nonwettable) cuticle, which has an external layer consisting of both cuticle microstructure and/or hydrophobic epicuticular waxes. The layer usually contains wax crystalloids, with dimensions ranging from hundreds of nanometers to micrometers (Fig. 13.6). The roughness of such surfaces together with their hydrophobic properties decreases wettability, which is reflected in a greater contact-angle of water droplets on such surfaces, compared to smooth surfaces of the same chemical composition. In some plant surfaces, this property results in their ability to be cleaned by rolling drops of water (Barthlott and Neinhuis, 1997, 1998). Similar insect structures, such as those on wings of representatives of Odonata, Ephemeroptera, and Neuroptera, are extremely nonwettable and self-cleaning (Wagner et al., 1996). In addition, these surfaces may prevent biofouling under water.

Many aquatic and semi-aquatic arthropods have sculptured surfaces involved in holding air underwater for respiration. Such surfaces, called plastrons usually contain fields of microtrichia, very small cuticle protuberances (Heckmann, 1983). These structures appear convergently in various arthropod taxa, as an adaptation to aquatic environments: Collembola, Lepidoptera, Coleoptera, Heteroptera, Diptera, Araneae, and Diplopoda (Thorpe and Crisp, 1947; Hinton, 1976; Messner, 1988). Some terrestrial insects, such as Aphididae (Auchenorrhyncha), also bear similar

structures in the form of bristles, mushroom-like spines, or stigmal plates, which can protect their surfaces from moisture (Heie, 1987). In water striders and some spiders, the anti-wetting surfaces of legs and the ventral body side are involved in the locomotion mechanism of walking on water.

Especially interesting are the genera *Halobates*, (Gerridae) and *Haloveloides* (Veliidae), the only open sea water dwellers (Perez Goodwyn, 2009). Representatives from the genus *Halobates* have outstanding water-protection structures. The microtrichia pile is composed of specialized prolongations. Each microtrichium has a thickened head, several times wider than the shaft, and usually tilted to one side like a golf club (Fig. 13.7). On the shaft itself, there are up to four perpendicular branches interlocking the microtrichia, the shafts of which are 0.8–1.1 μm apart. This structure provides effective water protection, in case of being submerged, a frequent occurrence in the open sea (Perez Goodwyn, 2009).

Surface outgrowths may provide multi-level reflection of sunlight. Such an ability of wing scales is suggested to be an adaptation for cooling in butterflies (Grodnicky, 1988). Body coverage by bristles, scales, and hairs in the honey-bee, *Apis mellifera*, may be used for warming up and influencing metabolism at low temperatures (Southwick, 1985). In species of curculionid beetles of the genus *Tychius* inhabiting arid areas, cuticular scales have been suggested to be a system responsible for maintaining thermal balance (Karasev, 1989). A water-loss preventing function was suggested for leaf-like bristles at the body margins in Aphididae (Auchenorrhyncha) (Heie, 1987). Surfaces of many desert insects are covered with hydrophobic wax-crystalloids that presumably decrease water evaporation through the cuticle and the particular pattern of hydrophobic and hydrophilic islands of the cuticle aids in water harvesting by condensation (Parker and Lawrence, 2001).

Highly specialized areas of cuticle, responsible for sound generation, usually consist of patterns of cuticular plates, seldom of microtrichia fields (Hinton, 1970). When these surfaces slide over each other, sound is generated. Such structures have

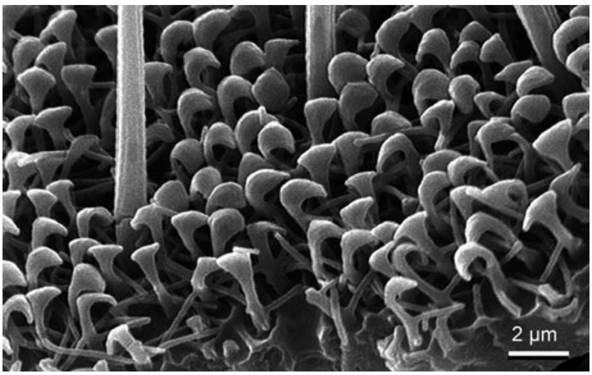


Fig. 13.7 Thoracic microtrichia cover of *Halobates germanicus* (Gerridae). Note golf-club-shaped heads and inter-locking extensions between them (Perez Goodwyn, 2009)

been previously described from elytra, abdomina and coxae in phylogenetically distantly related arthropod taxa: including bugs Cimicomorpha, Pentatomorpha, and beetles *Geotrupes* (Scarabaeoidea) (Gogala, 1984; Palestrini et al., 1987). The sound frequency corresponds to the periodicity of these structures contacting functionally corresponding surfaces, and the speed of sliding. These systems should be, in addition, wear-resistant. However, rigorous experiments supporting or rejecting this statement are absent in the literature.

Filtration systems are usually equipped with long bristles. Such systems are well-known from mouthparts of aquatic insect larvae. The filtering system of insect spiracles that keeps the tracheae free of dirt particles is composed of branched acanthae (Fig. 13.4g). However, in some cases, completely different principles may be involved in the design of these systems. The labellum of many flies (Brachycera, Diptera) bears so-called pseudotracheae (Gracham-Smith, 1930; Elzinga and Broce, 1986) whose outgrowths have a complex material structure. Labellum and pseudotracheae are driven by muscles, resilin springs, and hydraulic pressure, enabling a change in the diameter of the filtration sieve, depending on the size of the particles in the food (Fig. 13.4f).

Functional surfaces in technical systems have many functional requirements, which can be fulfilled by using ideas from biology. Figure 13.8 lists recent activities on the implementation of biologically inspired microstructure into product design. Since insects bear a huge variety of such microstructures, many of which have not even been previously described, a systematic approach to insect surface science would be very desirable. An important step in this direction is establishing a database of insect functional surfaces. Furthermore, we need more experimental studies, targeted to answering the relationship between structure at various levels of organization and their function. We believe that enhancing the pool of new ideas from biology will provide a leap in the surface technology of tomorrow.

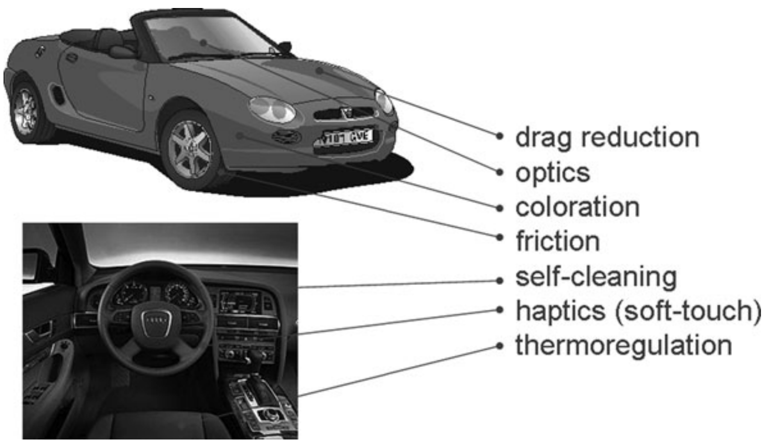


Fig. 13.8 Car-industry-related functional surfaces for which microstructure development uses ideas from biological studies on insects and some other organisms

13.4 Adhesives

There exist a variety of biological systems that prevent separation of two surfaces. These systems are often called *adhesives* or *attachment devices* (Gorb, 2001). Some of them are based entirely on mechanical principles, while others additionally rely on the chemistry of polymers and colloids (Scherge and Gorb, 2001; Habenicht, 2002). There are at least three reasons for using adhesives: (1) they join dissimilar materials; (2) they improve stress distribution in the joint; and (3) they increase design flexibility (Waite, 1983). These reasons are relevant both to the evolution of natural attachment systems and to the design of man-made joining materials.

In general, adhesive-bond formation consists of two phases: contact formation and generation of intrinsic adhesion forces across the joint (Naldrett, 1992). The action of the adhesive can be supported by mechanical interlocking between irregularities of the surfaces in contact. Increased surface roughness usually results in an increased strength of the adhesive joint in the glue-based systems due to the increased contact area between the contacting surfaces and the solidified adhesive substance. Strong adhesion is also possible between two ideally smooth surfaces. Adhesive organs, which may be used for attachment to substrates as well as being involved in catching prey, demonstrate a huge diversity among living organisms due to their structural and chemical properties. Biological adhesion underlies the organization of all living tissues. There is no doubt that many functional solutions have evolved independently in different lineages.

Many species of insects are supplied with diverse attachment devices, the morphology depending on the species' biology and particular function in which the attachment device is involved. The evolutionary background and animal behavior influence the specific composition of attachment systems in each particular species. There are eight fundamental classes of attachment principles found in insects: (1) hooks, (2) lock or snap, (3) clamp, (4) spacer, (5) suction, (6) expansion anchor, (7) adhesive secretions (glue), and (8) friction (Gorb, 2001). However, different combinations of these principles also occur in existing attachment structures. Three types of adhesion at the organism level are known: (1) temporary adhesion allowing an organism to attach strongly to the substrate and detach quickly when necessary (see the subsection below about locomotory attachment devices); (2) transitory adhesion permitting simultaneous attachment and movement along the substrate; (3) permanent adhesion involving the secretion of cement. These three types of adhesion do not have the same purpose and use different adhesive systems.

Walking machines usually use suckers to hold onto vertical and overhanging surfaces. A primary disadvantage of this attachment principle is that a very smooth substrate surface is required. A future goal is walking robots capable of walking on a variety of surfaces. Insects can walk rather well on smooth and structured substrata, on inclines, vertical surfaces, and some of them even on the ceiling. One such example is the hairy surface of legs in flies and beetles. This system uses a secretion enabling hairs to attach and detach very quickly to diverse substrata. The hair design includes a mechanism that delivers the secretion, in extremely small

amounts, directly to the contact area (Ishii, 1987), and only then when contact to the substrate is achieved.

Generally, in their evolution, insects have developed two distinctly different mechanisms to attach themselves to a variety of substrates: with smooth pads or with setose, or hairy surfaces. Because of the flexibility of the material of the attachment structures, both mechanisms can maximize the possible contact area with the substrate, regardless of their microsculpture. Tenent setae are relatively soft structures (Niederegger et al., 2002). In *Calliphora* flies, their tips are usually compressed, widened, and bent at an angle of about 60° to the hair shaft (Bauchhenss and Renner, 1977). Some male beetles possess mushroom-like terminal tips of setae (Fig. 13.9a).

Different forces may contribute to the resulting attachment force: capillary adhesion and inter-molecular van der Waals forces. A contribution of inter-molecular interaction to the overall adhesion has been shown in experiments on the adherence of beetles (Stork, 1980), and beetle setae (Stork, 1983) on a glass surface. The presence of claws, decrease of air pressure, decrease of relative humidity, or electrostatic forces do not influence beetle attachment on smooth substrata. In the beetle *Chrysolina polita* (Chrysomelidae), the resulting attachment force directly depends

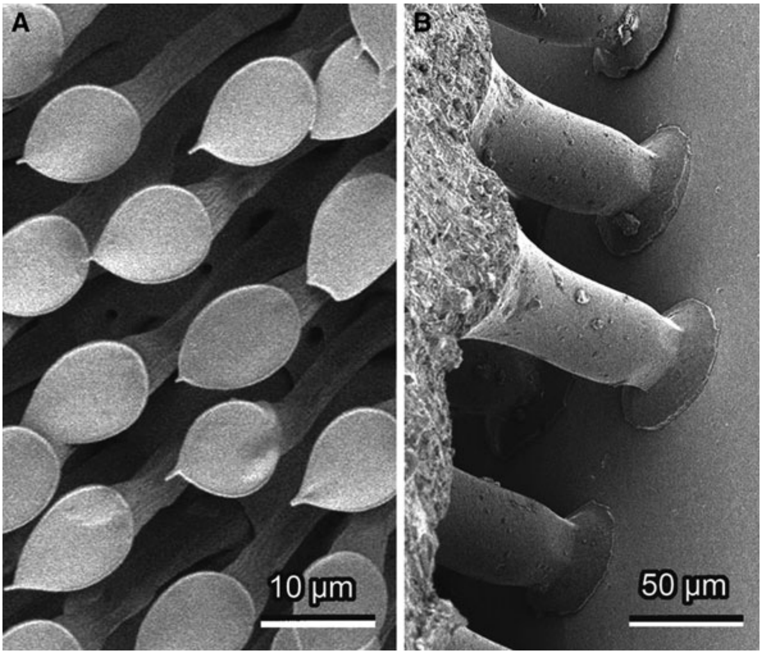


Fig. 13.9 Insect-inspired adhesive. **a** Ventral surface of the tarsus in the male of the chrysomelid beetle *Gastrophysa viridula*. **b** Microstructured polymer foil adhering to a glass surface. Note mushroom-shaped geometry of terminal contact elements in both systems (Gorb et al., 2007)

on the number of single hairs contacting the surface. Recently, the contribution of inter-molecular interaction and capillary force has been demonstrated for the fly *Calliphora vicina* in a nano-scale experiment using an atomic force microscope (Langer et al., 2004). Attachment forces increase when the contacting surfaces slide against each other. This may explain why flies placed on a smooth under-surface always move their legs in a lateral-medial direction (Wigglesworth, 1987; Niederegger and Gorb, 2003). During these movements, setae slide over the surface obtaining optimal contact.

The size of single points in hairy attachment devices gets smaller and their density higher as the body mass increases (Scherge and Gorb, 2001; Arzt et al., 2003). The fundamental importance of contact splitting for adhesion on smooth and rough substrata has been explained by a very small effective elastic modulus of the fiber array (Persson, 2003). Adhesion enhancement by surface micropatterning has also been demonstrated experimentally (Peressadko and Gorb, 2004; Gorb et al., 2007). A patterned surface, made out of polyvinylsiloxane (PVS), has significantly higher adhesion on a glass surface than a smooth sample made out of the same material (Fig. 13.9b). An additional advantage of patterned surfaces is the reliability of contact on various surface profiles and the increased defect tolerance of individual contacts.

Hairy leg attachment pads are promising candidates for biomimetics of robot soles adapted for locomotion. Similar principles can be applied to the design of micro-gripper mechanisms with an ability to adapt to a variety of surface profiles (see the section *Robotics* in this Chapter). There are very interesting insect adhesive systems combining microstructure and glue, such as the mouthpart apparatus used by rove beetles (genus *Stenus*) to trap the animals they feed on (Betz et al., 2009). The beetles' extended, rod-shaped lower lips have two sticky pads which shoot out like a catapult to capture potential prey (Fig. 13.10a, b). This system is currently a topic of several biomimetic projects. The first stage in developing rove-beetle-inspired technical adhesives is to analyze the key structural and biomechanical data (Fig. 13.10c). It seems that the adhesive function is not only based on the subdivision of the contact area into many smaller ones, but also on very special features of the beetle glue itself: (a) fibrillation at the surfaces' separation (energy dissipation mechanism and crack propagation preventing mechanism), (b) biphasic nature of the glue (universal wettability mechanism), (c) rapid change of its viscosity (viscose force adhesion mechanism).

The adhesives industry is presently following three main goals (Hennemann, 2000): (1) an increase in the reliability of the glued contact; (2) mimicking of natural, environment-friendly glues; (3) development of mechanisms for application of a minute amount of glue to the surface. An additional challenge is the use of substances and/or mechanisms that allow multiple attachments and detachments, and enable attachment to a variety of surfaces. The present section clearly demonstrates the strong potential of insect adhesives for inspiring development of technical adhesives that fulfill the listed requirements.

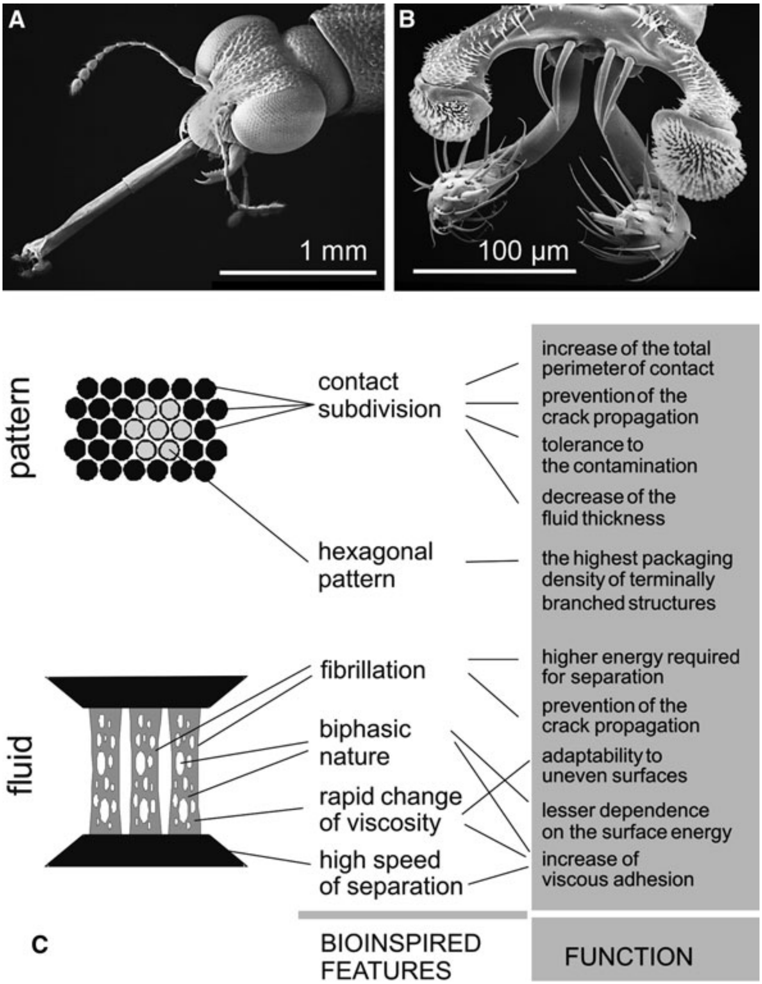


Fig. 13.10 The adhesive capture apparatus of the rove beetle *Stenus comma*. **a** SEM image of the head with the protruding lower lip (*labium*). **b** SEM image of the tip of the labium with the adhesive pads. **c** Summary of the functional principles with biomimetic potential in the system under investigation (Betz et al., 2009). **a**, **b** Courtesy of O. Betz (University of Tübingen, Germany); **c** Original

13.5 Optics

Ommatidial gratings are anti-reflective structures on the eyes of insects, especially those which are nocturnally active (Fig. 13.11). These protuberances are very small microtrichia (200 nm in diameter), which increase visual efficiency through decreased surface reflection in their density, and increased photon capture for a given stimulus condition (Parker et al., 1998; Vukusic and Sambles, 2003).

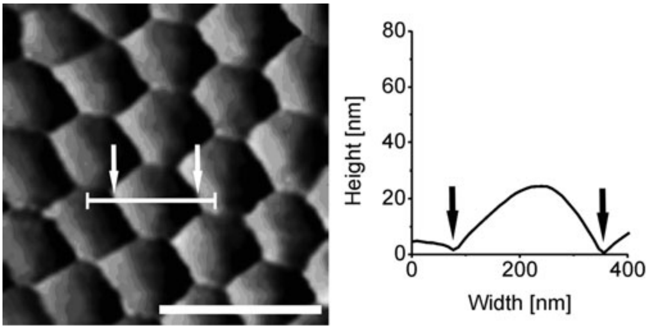


Fig. 13.11 Atomic force microscopic (AFM) error channel image (*left*) and profile (*right*) of ommatidia of the moth *Laothoe populi*. The *white bar* marks the region used for measurements of surface profile. The grating borders indicated by the *arrows* in the left picture correspond to the *arrows* shown in the surface profile diagrams. *Scale bar* = 500 nm (Peisker and Gorb, 2010)

Such a grating is particularly useful on a curved corneal surface, since it increases the transmission of incident light through the cornea compared with a smooth surface. For an increase in transmission and reduced reflection, a continuous matching of the refractive indexes n_1 and n_2 at the boundary of both adjacent materials is very critical. If the periodicity l_1 of the grating is smaller than the wavelength l_2 of transmitting light, only light of zero order can be reflected or transmitted. For a constant ratio of both materials (cuticle-air) at the boundary between media, the electromagnetic field strength of incoming light is nearly constant (Bernhard et al., 1965). This region, therefore, can be considered as homogenous and an effective refractive index can be given.

Grooming is a very important function for insects that sometimes live in extremely dirty or dusty environments. Their rich sensory equipment of eyes and antennae has to be kept clean in order to respond adequately to external signals. Many insects bear specialized cleaning structures (Schönitzer and Lawitzky, 1987; Francouer and Loiselle, 1988). Others rely on the micro- and nano-structured surfaces with an anti-adhesive function. Ommatidia gratings are a multi-functional surface that employs self-cleaning due to the real contact surface reduction mechanism (Peisker and Gorb, 2010).

13.6 Photonics

Structural coloration, due to the presence of scales and bristles, is well known in insects, such as butterflies (Ghiradella, 1989) and beetles (Schultz and Hadley, 1987). For example, scales of some scarabaeid beetles bear additional microtrichia on their surfaces responsible for the lusterless appearance of the elytra surface (Fig. 13.4c, d). The coloration pattern serves for species and sex recognition, and also for camouflage and mimicry. The most interesting type of structural coloration

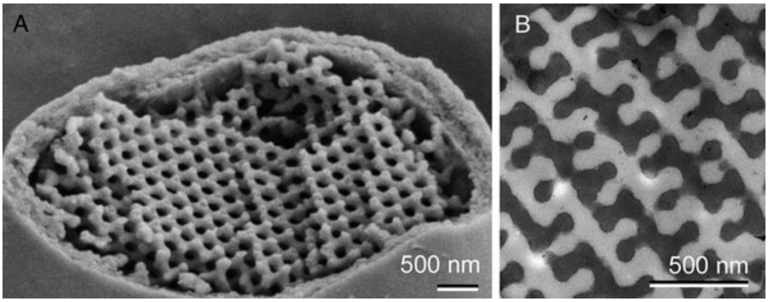


Fig. 13.12 Photonic crystals of the dorsal scale-like hairs in the curculionid beetle *Phyllobius argentatus*. **a** SEM micrograph of the cross fracture through the scale. **b** TEM micrograph of the oblique ultrathin section of the scale

is called iridescence, which is well known in insects and birds, and has been characterized for many different species (Ghiradella et al., 1972; Huxley, 1975).

The iridescence is a result of optical interference within multi-layer structures (Ghiradella, 1991) that are rather complex in their architecture, and may be incorporated into systems that can produce several different optical effects. Such effects include diffraction-assisted reflection angle broadening (Vukusic et al., 1999, 2000a), all-structural color mixing, and strong polarization effects (Vukusic et al., 2000b) (Fig. 13.12).

13.7 Sensorics

Insects carry out flow sensing in various contexts using mechanosensory hairs (Gnatzy and Heusslein, 1986). The wide variety in hair length and number in insects and other arthropods can be partly explained by the physics of the medium and its interactions with the hair (Humphrey et al., 1993, 2003). For example, filiform hairs provide crickets with a highly sensitive receptor system capable of differentiating air-particle movements in the low-frequency range due to their diverse combinations of directional selectivity, sensitivity and frequency response.

Crickets also possess composite mechanoreceptors. For example, the coupling of filiform hairs with campaniform sensilla creates a mechanoreceptor with an extended working range (Heusslein et al., 2009) (Fig. 13.13). In addition, the composite mechanoreceptor serves as a tactile receptor system superior in its spatial detection range.

The arrangement of hairs also has important sensory effects (Casas and Dangles, 2010). The spatially heterogeneous information provided by a transverse flow around a cylinder implies that hairs should be placed all around a cylinder, maximizing the chances to perceive a source coming from any angle (Steinmann et al., 2006). Cricket air flow sensors have recently been a source of inspiration for building

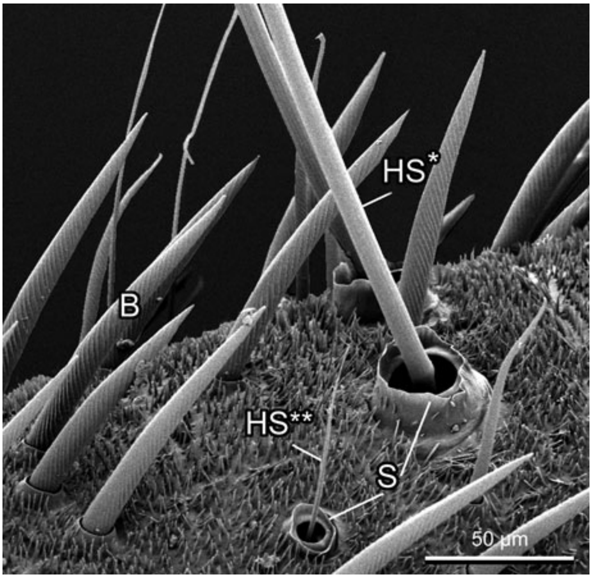


Fig. 13.13 SEM of cercal filiform hairs and campaniform sensilla of the cricket *Gryllus bimaculatus*. Cercus surface showing a filiform hair with a long hair shaft (*HS**) inserted in a large cuticular socket (S) and a filiform hair with a very short hair shaft (*HS***) inserted in a small socket. B, bristles (Heusslein et al., 2009)

artificial air flow sensors (Dijkstra et al., 2005). Design guidelines for building flow-sensing MEMS arrays were also based on biomimetic ideas from studies on the cricket's cerci. A spatial arrangement of MEMS hairs with a large range of angles relative to flow direction on a dedicated platform increases the sensitivity of such sensors. Such design could represent a major advance in the actual mounting of MEMS hairs on a horizontal plate. This is, however, not a trivial task in MEMS fabrication.

13.8 Robotics

Insect-inspired robotics has emerged in the last decade. Because insects are structurally and functionally complex, a complete technical copy of an animal in hardware and software is not possible (Delcomyn, 2004, 2007). There are two different opinions on this matter. Some researchers suggest that as many features of an animal as possible should be incorporated into a robot (Ritzmann et al., 2000, 2004). Other researchers argue that including too many animal-like features into a robot can even impair performance (Yoneda and Ota, 2003). Which features of insects and which aspects of their locomotion are really important for implementation into robot design?

One significant contribution to robotics from entomology can be the search for non-conventional ways to move parts of the body for example due to specialized joints, springs, and latches. Work is already in progress on a variety of novel actuators, such as electro-elastomers (Pei et al., 2003) and ionic polymeric-conductor composites (Shahinpoor, 2003) and it seems likely that even more will be developed in the near future. A second contribution is the concept of dynamic stability allowing insects to run with high speed and strong maneuverability. Especially challenging is locomotion on the wall and ceiling (Daltorio et al., 2005a, 2005b, 2007, 2009). Generally, an incorporation of biomechanical principles into robots has recently contributed to the better performance of these robots (Altendorfer et al., 2001; Witte et al., 2004). The sensory feedback is critical to a fully functional,

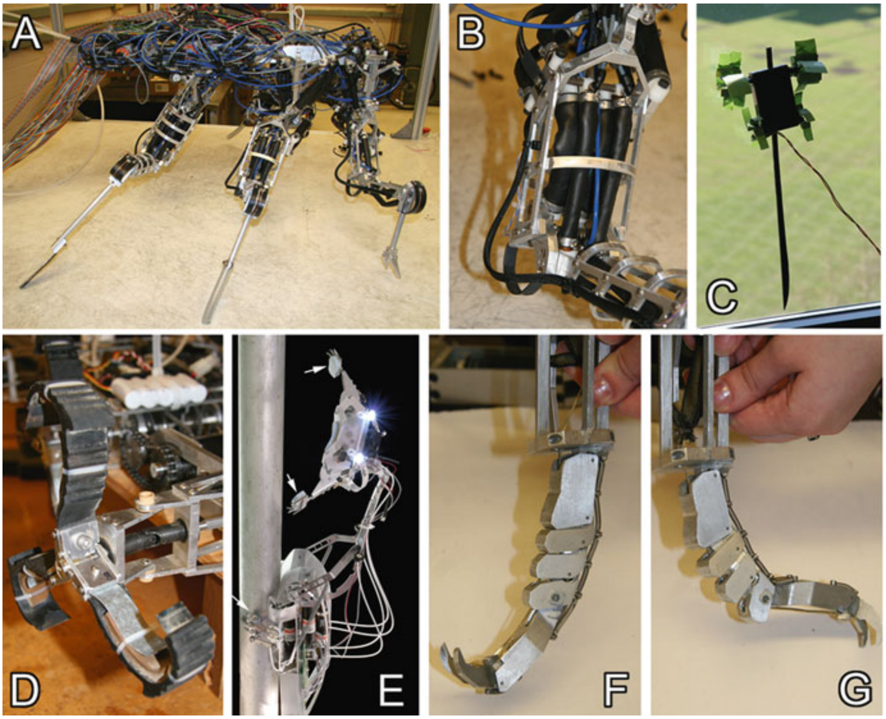


Fig. 13.14 Some examples of insect-inspired robots. **a**, **b** Robot with hexapod kinematics based on studies of the cockroach performed in the Ritzmann Lab in the Biology Department at Case Western Reserve University (CWRU, Cleveland, USA). It has a total of 24° of freedom with five for each front leg, four for the middle legs, and three for the rear legs. **c** Climbing Mini-Whegs^{TM} scales vertical glass surfaces using compliant, adhesive feet (Daltorio et al., 2005a, b, 2007, 2009). Like all Mini-Whegs^{TM} robots, it has one drive motor that turns all four wheel-legs in an alternating diagonal gait. **e** InspiRatnik robot based on the principle of locomotion of geometrid caterpillars. **f**–**g** Gripping leg of the robot inspired by an insect tarsus. **a**–**d**, **f**–**g**. Robots built in the Quinn Lab (CWRU, Cleveland, USA). **e** Robot built by TETRA GmbH (Ilmenau, Germany)

agile walking robot (Schmitz et al., 2001). This is the least developed area of bio-inspired robotics. The reason is that it is difficult to make light but effective artificial sensors. Finally, central control of early robots must be changed to more distributed control that is typical for biological systems including insects (Delcomyn, 1999).

Figure 13.14 shows some implementations of insect-based research in robotic systems. Many labs worldwide have designed hexapod robots, but probably the most elaborate ones originate from the Quinn Lab at Case Western Reserve University (Cleveland, USA) (Fig. 13.14a, b). The Prolero, RHex, and Whegs™ series robots utilize a method of locomotion that combines the advantages of wheels and legs (wheel-legs). Wheels are relatively simple, and allow a vehicle to move over terrain quickly. Legs allow robots to climb obstacles that are higher than what a wheeled vehicle would be able to climb over. These robots equipped with adhesive materials are capable of climbing up a glass wall (Fig. 13.14c, d). Structural data on cockroach legs (Frazier et al., 1999) were used to machine insect legs from aluminum blanks, in order to mimic chain-like tarsi capable of optimal grip with various kinds of terrain (Fig. 13.14f, g). Caterpillar-like locomotion has inspired design of the InspiRatnik robot that was recently tested by the company TETRA GmbH (Ilmenau, Germany).

13.9 Future Perspectives

What can be done to advance the field of insect-inspired biomimetics? First, and obviously, additional research on insects will help, not just in the application of biological knowledge to engineering problems, but on the biological systems themselves. Incorporating more biological knowledge into the design of artificial systems will improve their performance, but the fact of the matter is that biologists still do not have a complete understanding of how insect materials are constructed, what their performance is, how insect locomotion is generated, controlled, and regulated, etc. Hence, many technological areas will benefit from additional entomological research.

Additionally, a huge variety of insects and their systems have not been previously studied at all. That is why screening for new interesting systems seems to remain an extremely important research field in the nearest future.

Only a few examples of bio-inspired systems have been tested in order to compare their performance in comparison with conventional systems or in comparison with their biological prototypes. The field of biomimetics in general would benefit greatly from development of a set of standard tests that can be used to evaluate the performance of bio-inspired materials and systems. The objective of biomimetics is to improve the performance of biologically inspired systems in the real world. Then it appears to be essential that these systems have to be subjected to real tests of performance. Clearly, the kind of tests will vary from system to system and be different for systems that have different performance objectives.

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